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**COLD SPRING HARBOR SYMPOSIA
ON QUANTITATIVE BIOLOGY**

VOLUME IX

COLD SPRING HARBOR SYMPOSIA
ON QUANTITATIVE BIOLOGY

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COLD SPRING HARBOR SYMPOSIA
ON QUANTITATIVE BIOLOGY

VOLUME IX

Genes and Chromosomes
Structure and Organization

THE BIOLOGICAL LABORATORY

COLD SPRING HARBOR, L.I., NEW YORK

1941

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FOREWORD

When it was decided that this year's Symposium would deal with a problem integrating genetics and the borderline fields of physics, chemistry and mathematics, it seemed logical to select "Genes and Chromosomes—Structure and Organization" as the topic of the symposium. Mathematics is regularly used in the analysis of genetic problems so that whichever topic were selected would have included mathematics. For a number of years, in the studies dealing with the structure of genes and chromosomes physical and chemical methods have been extensively employed, and physical and chemical interpretations have been utilized. At present the work on these problems is at a stage where an organized discussion with participants representing borderline fields seemed profitable.

Since 1936 a small group of biologists, biophysicists and biochemists interested in the gene problem has been holding conferences at regular intervals. This group made up the nucleus of the 1941 Symposium, and the Symposium itself was an expanded gene conference.

In discussions between geneticists and physicists among the questions invariably asked are: What is the approximate thickness of the chromosome threads; when do they divide; how close together do they lie; how tightly are they coiled; and what is their number? The experimental evidence pertaining to these and related questions was presented in the first section of the Symposium dealing with the "Structure of chromosomes as revealed by optical methods." This gave an outline of the known facts which were useful in discussion of problems brought out later.

It is generally assumed that in giant salivary gland chromosomes found in the larvae of flies the primary chromosome thread is multiplied a great many times. The structures which are visible in salivary gland chromosomes are also present in the chromosomes of other cells but they cannot be detected since they are too fine for our microscopes. Thus salivary chromosomes constitute material unusually suitable for

studies of fine structures and for the study of changes induced in chromosomes. Known facts dealing with the problems in which salivary chromosomes were utilized were discussed in the second section of the Symposium.

The third section dealt with the "Spontaneous and induced changes in chromosome structure." It is known that chromosomes break spontaneously and also that such breaks may readily be induced by X-rays and similar radiations. Since the occurrence of a break is undoubtedly connected with some chemical reaction, the studies of the breaks may give a clue for an analysis of the chemical properties of chromosomes.

Spontaneous and induced changes in genes were discussed in the fourth section of the Symposium which was designated as "Mutations." An emphasis was placed on the problem of spontaneous mutations which has lately been neglected in similar considerations. It is felt that data on spontaneous mutations may help to clarify certain problems dealing with the induced changes and thus may contribute toward better understanding of chemical processes involved in mutational changes. However a full opportunity was afforded for discussion of changes in genes induced by various physical agents, since at present these data constitute the best material for interpreting the physical and the chemical properties of genes and chromosomes.

"Physical aspects and tools" were discussed in the fifth section of the Symposium. New tools like the electron microscope may well prove an important factor in the study of properties of very fine structures such as chromosomes. Thus the information about the electron microscope may accelerate the work in that field. For interpretation of changes induced in genes and chromosomes by radiation, familiarity with certain physical aspects is essential.

It seems very probable that genes are large organic molecules in which protein and nucleic acid are present. Thus a discussion of the prop-

erties of giant molecules and particularly of proteins, nucleic acid and viruses were topics which logically belonged in this Symposium. Attention of the group was called to the evidence which indicates that frequent atomic interchanges occur in living organic molecules since this may have an important bearing on the visualization of the activity of a gene. A general résumé of ideas brought out in the Symposium was presented as a concluding lecture.

Previous Symposia lasted for five weeks and the majority of participants remained in residence at the Laboratory for a part of that time. This year the program containing approximately the same number of papers was condensed into two weeks. Such modification helped a great deal in keeping the group together and the majority of participants remained in residence during the whole session of the Symposium.

It has been generally assumed that in order that a symposium be a success the attendance should be limited to a relatively small group, since it is believed that a large group hampers the free discussion which is an essential part of

a symposium. It has been the policy of the Laboratory to have the Symposia open to all who desire to attend, and this policy was followed this year. I was rather disturbed when instead of the expected attendance of 35 to 50 persons, we had an attendance of about 120. However, as soon as the first session was over, it was evident that the large attendance not only did not prevent free discussion, but that it actually stimulated it. Our Symposium this summer has demonstrated that the interest and not the size of the group determines the success of a conference.

Discussions were recorded by Doctor Katherine S. Brehme and the first draft of the manuscript was prepared after consultation with the participants. After the first draft was revised by those taking part in the discussions, the second draft was prepared and circulated for revisions. The final manuscript was prepared from the second revision of the draft. Thus the discussions should give a true picture of the opinion of the group at the time this volume was written. The volume was edited by Doctor Katherine S. Brehme.

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CHROMOSOME CONTINUITY AND INDIVIDUALITY

H. E. WARMKE

In this introductory paper I should like to review briefly the fundamental concepts of cytogenetics and some of the original experimental evidence upon which these rest, before entering into the subject I was asked to discuss, that of external chromosome structure. I should like to start by mentioning some drawings which Hofmeister made from living cells of *Tradescantia* in 1848, very close to one hundred years ago. These, of course, were among the beginnings of nuclear cytology; I recall them here to remind you that the science of chromosomes is not exactly an infant, although it is very young in contrast to some of the other sciences represented here at the Symposium. Hofmeister, as is shown by his figures, clearly observed that the nucleus of the spore mother cell resolves itself into bodies (which we now call chromosomes) and that these bodies separate in a definite manner so as to take part in the formation of the daughter nuclei. In 1848, of course, the significance of these structures in heredity was not known; the figures were purely descriptive. These early observations of Hofmeister and others on living cells, however, constitute what I should like to include as first among seven fundamental discoveries in cytology, 1) that *the cell nucleus may resolve itself into microscopically discrete bodies, which we call chromosomes.*

The perfection of histological techniques and the compound microscope in the latter half of the 19th century ushered in the next important steps in cytological advance. With the introduction of refinements, such as the killing and fixing, sectioning, staining, dehydrating, and mounting of tissues, it was possible to observe more minute and delicate structures. During this period the second important discovery in nuclear cytology was made, 2) that *somatic cell division is accomplished by a process in which the chromosomes split lengthwise and the identical halves are so distributed that each of the two daughter cells receives the same number and kind of chromosomes which the parent cell contained.* It was shown by Flemming and Strasburger in 1882 that the chromatic threads split lengthwise early in cell division. Van Beneden almost immediately followed up this discovery with the observation that the identical halves of these split chromatic threads separate and pass to opposite poles at anaphase, and each is subsequently incorporated in one of the two newly-formed daughter nuclei. The important process of mitosis was thus described and understood.

Improved microscopic technique was also largely responsible for the next fundamental advance in cytological knowledge, 3) that *sexual reproduction is characterized by the union of the reduced nuclei of male and female gametes to form the primary nu-*

cleus of the embryo, which as a consequence of such union has the somatic number of chromosomes. It had been known for a long time that in some way or other the presence of a male gamete was required to initiate development in the egg. It was chiefly the researches of Hertwig (1875) and Strasburger (1877), however, which showed that the primary nucleus of the embryo results from the union of two nuclei, one from the egg and the other contributed by the sperm. The brilliant researches of Van Beneden (1883, 1887) on the round worm, *Ascaris*, completed the picture by showing that the chromosome number is reduced to one half in sperm and eggs and that at fertilization the nuclei of the egg and sperm contribute equally to the chromosome constitution of the offspring and restore the somatic number.

The rediscovery of Mendel's laws in 1900 gave new direction and new impetus to cytological investigations. This called forth what we might designate as the beginnings of true cytogenetics. Sutton, DeVries, and others called attention to the fact that the behavior of chromosomes offered a mechanical explanation of Mendel's laws. The fertilized egg and the organism which develops from it have two sets of chromosomes, one of maternal and one of paternal origin; Mendel had shown that a pea plant behaves as if each cell contained two sets of hereditary units. In eggs and sperm, only one set of chromosomes is present; in like manner, gametes were shown to behave as if they contained only one set of hereditary units. It was the beautiful work of Boveri (1909), however, demonstrating 4) that *chromosomes maintain a physical and genetic continuity through successive cell generations*, which placed the chromosome theory of heredity on a factual basis.

It is difficult or impossible, even today, to identify individual chromosomes throughout a complete division cycle. During the resting stage the identity of chromosomes is lost to view, and the nucleus appears to be filled with a mass of anastomosing strands or fibers. The laws of heredity require a genetic continuity, not only through a complete division cycle, but through the scores of these divisions between the egg and adult, and any structure assumed to be the carrier of the genetic units would therefore have to maintain physical continuity through successive division cycles. Boveri showed that chromosomes reappear at prophase in the same relative positions they occupied at the preceding telophase, in the early cleavage stages of *Ascaris*; he thus presented strong experimental evidence that even though chromosomes may be lost to view during certain stages, they do maintain physical continuity throughout successive division cycles.

Workers in this early period generally assumed that all the chromosomes in a cell were alike, both morphologically and genetically. New genetic theory, however, held that only two genes of a kind exist in a somatic cell and only one in a gamete; if chromosomes were to be considered the bearers of genes, this meant that no more than two chromosomes in the diploid organism could be alike genetically, regardless of the total chromosome number.

It was Boveri who established another basic chromosome concept when he showed, 5) that *the chromosomes of a complement differ qualitatively*. It was known that occasionally a sea-urchin egg is fertilized by two sperms, and that when this occurs the first cleavage is multipolar, and the egg generally is divided into four cells. Subsequent cleavage divisions are bipolar and quite normal, but as a rule, development leads to the production of a variety of forms with structural abnormalities, and most of such larvae fail to survive.

Boveri observed that there is an irregular distribution of chromosomes of the multipolar division and that the cells resulting from this abnormal division rarely have the same number of chromosomes. It was not the number of chromosomes or total amount of chromatin that caused abnormal development, however, because eggs containing the haploid, diploid, triploid, and tetraploid number of chromosomes were known to develop essentially normally. Nor was it an unequal division of the cytoplasm that caused abnormal development, for in the quadri-polar cases four equal sized cells are formed, exactly as in the normal embryo at the end of second cleavage. Boveri (1902) therefore concluded that "normal development is dependent on the normal combination of chromosomes, and that this can only mean that individual chromosomes must possess different qualities."

This concept has been confirmed in various other ways since Boveri, notably by Blakeslee and his group, who showed that *Datura* plants with single extra chromosomes ($2n + 1$ types) differ in appearance depending on which one of the twelve chromosomes is the extra one.

The next concept, 6) that *the hereditary units, or genes, are arranged in constant linear order within the chromosomes* dates back at least to Roux (1883). Genetic data and the elongate nature of the chromosomes, especially during meiotic prophase when small enlargements, the chromomeres, are seen in a linear order along the chromatic threads, made it logical to assume that genes are arranged as beads on a string. The final cytological proof, however, did not come until much later, when the linear correspondence between genetic loci and bands of the salivary gland chromosomes was shown by Painter, Bridges and others.

The last basic concept I should like to bring before you is 7) that *the hereditary units are linked together in groups equal to the number of chromosome pairs, but exchange of segments of genes may*

take place between homologous chromosomes by the process of crossing over. It soon became evident, after genetic experiments increased in number, that Mendel's principle of independent assortment did not hold in all cases, but that certain groups of factors tended to remain in the combinations in which they entered; thus to be linked. It was mainly through the research of Morgan and his associates that it was made clear that those genes located within the same chromosome did not assort independently, but were linked; the number of such linkage groups being equal in number to the haploid chromosome number. Genetic evidence, however, indicated that there was occasional exchange of segments of homologous linkage groups; this was called "crossing over." This should be accompanied by an exchange of segments of homologous chromosomes, but since homologous chromosomes are normally identical in size and shape an exchange of segments could not be detected under the microscope. Recently Stern on *Drosophila* and Creighton and McClintock on maize, using translocation stocks in which it was possible to distinguish the two members of a pair of homologous chromosomes cytologically, were able to show that when an exchange of segments of linked genes takes place that there is also a corresponding exchange of segments of homologous chromosomes.

I have not attempted to document this brief review adequately (see Wilson, 1925 and Sharp, 1934 for more detailed treatments and references); also the classification of the fundamental concepts has been largely arbitrary: they might have been expanded into more or contracted into fewer principles, but I think most of the important points have been covered. These principles may not be mentioned again, as such, during the course of the entire symposium; nevertheless, they will underlie the whole program—they will be taken for granted for the most part. I thought it might be well here in the very beginning to review and reaffirm these basic concepts before branching out into some of the newer and more specialized fields of investigation, which will occupy the attention of the symposium for the next two weeks.

I should now like to briefly review some of the more recent knowledge of chromosomes—specifically, what we see under the microscope, using modern techniques.

Chromosome Shape: Chromosomes are more or less rod-shaped bodies but change shape widely depending on the phase of the division cycle. Let us start with chromosomes as they appear at metaphase or anaphase of mitosis. At this stage the nuclear membrane has broken down, and the chromosomes lie in the cytoplasm as sausages. The main body of the chromosome at this stage is made up of a ground-substance called the matrix; and twisted or variously coiled within the matrix lie the chromatic threads or chromonemata, which are the effective bearers of heredity. The outer boundary of the

chromosome proper is called the pellicle by some workers. I must not say more about the chromonemata, because these are the subject of the next two Symposium papers; I am supposed to stay outside of the chromosome. Perhaps I should say, however, that the chromonemata may be revealed microscopically by proper techniques, and also that the pellicle and matrix more or less completely disappear during certain stages of division, thus exposing the naked chromatic threads.

Chromosomes at metaphase and anaphase are usually not just straight rods; they ordinarily have two arms, which may or may not be equal in length. At the point of junction of the two arms the chromosome is usually constricted into what is known as the centric or attachment constriction. The centromere or kinetochore is located at this point of constriction, and may be demonstrated by proper techniques in certain species as small, spherical bodies in the chromatic threads. Navashin has figured them in *Galtonia*, Trankowsky in *Crepis* and *Najas*, and Schrader in the amphibian *Amphiuma*. In most species they cannot be demonstrated cytologically, but are assumed to be present by the behavior of the chromosomes. The centromere is clearly visible in the pachytene stages of corn, as McClintock (1930) and others have shown. Here the centromere appears as a small transparent sphere connecting the chromatic threads of the arms.

Each chromatid normally has a single centromere. If two centromeres are present in a single chromatid as they may be, for example, after crossing over in an inversion, a dicentric is formed. A dicentric usually arranges itself so that the two centromeres go to opposite poles; it thus forms a bridge across the cell at anaphase, which is resolved only by breakage of the thread. If no centromere is present, as is the case with so-called acentric fragments, the chromatid does not align itself properly on the spindle and usually is lost during cell division.

The centromere is an extremely important structure. It is the interaction of the centromere with the centrosome of animal cells or with the spindle poles of plant cells which orients the chromosomes on the metaphase plate. This is shown by the behavior of acentric fragments, which are usually left off the plate at metaphase and lying out in the cytoplasm; while centric fragments and whole chromosomes behave in the usual manner. The centromeres are also of great importance in initiating separation and poleward migration of the chromatids at anaphase. Separation of the chromatids at anaphase begins at the centromere and proceeds, with the centromere leading the way, until separation is complete and until the chromatids reach the poles.

The position of the centromere along the chromatic thread determines the characteristic shape of the chromosome at anaphase. Whether the chromosome is V, J, or I-shaped will depend upon the relative lengths of the arms—whether the centromere is median, submedian, sub-terminal, or possi-

bly terminal. Terminal centromeres are probably rare or absent in nature; they are known in experimental strains of maize (Rhoades, 1940) and probably also in *Melandrium*.

The recent work of Pollister in correlating loss of centromeres with supernumerary centrosomes is extremely interesting. In certain snails exceptional, nonfunctional sperms are produced; Pollister (1939) has shown that the centromeres of some or all of the chromosomes are missing and that these chromosomes behave as acentrics at meiosis. In the same cells he has observed supernumerary centrosomes, equal in number to the chromosomes without centromeres. This suggests a definite relationship between centromere and centrosome and may lead to some sort of an explanation of the poleward migration of chromosomes.

Chromosome shape is also modified by secondary constrictions. These resemble centric constrictions, but do not have centromeres, and are in addition to the centric or primary constriction. A good example of secondary constriction is found in *Vicia*, where such constrictions are found in one or two pairs of chromosomes, depending upon the species (Heitz, 1931). It is probably also correct to consider satellites, those small bead-like bodies attached usually to the ends of chromosomes by a thread, as separated from the main body of the chromosome by a secondary constriction.

Secondary constrictions provide distinctive landmarks to identify certain chromosomes, and also play an important role in nucleolus formation. Navashin (1927), Heitz (1931), and others have shown a definite and constant relationship between secondary constrictions, including satellite constrictions, and the formation of nucleoli.

Chromosome ends are also differentiated to some degree, although there is no visible structure. Experimentally broken chromosome ends tend to fuse with other broken ends to form translocations, inversions, etc., or with themselves to produce chromatid bridges; normal chromosome ends do not unite with other ends to form chromosome chains or fuse to form bridges at anaphase, nor can broken ends be made to attach to normal ends.

The chromosome, then, is a differentiated unit, with a centromere connecting two arms of varying lengths and having autonomous ends. We should not come to think of chromosomes as simply segments of chromatin, but rather as highly integrated and differentiated units.

Chromosome Size: It is difficult to get accurate measurements of chromosome dimensions: First, because length and breadth vary with the stage of the division cycle; they are long at prophase and shorter at metaphase, and it is difficult to draw a sharp line between stages. Secondly, because different killing and fixing agents and different conditions of growth cause different degrees of shrinkage or swelling of chromosomes. Thirdly, because chromosome size may be under genic control as has been

shown by Lesley and Frost (1927). These workers found that chromosomes at MI in a certain strain of *Matthiola* were considerably shorter than in others. Breeding experiments showed that chromosome size in these lines was genetically controlled, short chromosomes behaving as a simple mendelian dominant to long chromosomes. All these sources of variation probably hinge upon the fact that the chromonemata of chromosomes at metaphase are coiled, with the degree of contraction determining the relative length and width of the overall structure.

Nevertheless, averages give a fair idea of the range in size of chromosomes encountered in different species. The chromosomes of the fungi, for example, are generally small; the average size of the spherical chromosomes in *Saprolegnia*, according to Mäkel (1928) is less than 0.5 microns. In *Trillium*, on the other hand, the largest chromosome of the complement averages some 30 microns in length by three microns in width in somatic divisions (Warmke, 1937). Most forms have chromosomes intermediate in size. Maize chromosomes average eight to ten microns in length; the longest chromosomes in *Drosophila melanogaster* are about 3.5 microns. *Tradescantia* chromosomes average about 10 microns; *Melandrium* two to six microns (Warmke and Blakeslee, 1940); and *Datura* 1.5 to four microns (Satina, Bergner, and Blakeslee, 1941). There may be a considerable range in size of chromosomes in the same complement. In *Drosophila* the second and third chromosomes are about 3.5 microns in length, but the fourth is only 0.3 microns. In *Yucca* there is also a wide range, five pairs are long, about six microns, and 25 pairs small, about one micron (McKelvey and Sax, 1933).

Chromosome Number: The number of chromosomes in a complement, as the size of chromosomes in a complement, is extremely variable in different species, but is normally constant within a species. The lowest chromosome number known and the lowest possible in a sexually reproducing form is $n = 1$, $2n = 2$, which is found in the round worm, *Ascaris megalocephala* univalens (Boveri, 1909). This example is probably not a very good one because there is the complication of a fragmentation of the chromosomes in somatic cells to form a larger number. *Crepis capillaris* and several species of *Crocus*, however, have a haploid number of three. Chromosome numbers range from these low ones up to several hundreds. In a summary of almost 2500 species of plants, Fernandes (1931) found that the haploid numbers 12, 8, 7, 9, 16, 6, 10, 14 occurred most frequently, and in that order.

Chromosome numbers are ordinarily constant; a given species will have the same chromosome number regardless of where it is found. There is the phenomenon of polyploidy, however, which is especially common among the plants, where two individuals of the same species or more commonly, two closely related species may have chromosome numbers which differ by a simple multiple of the basic num-

ber. Thus, one variety or species may have a haploid chromosome number of nine, and closely related forms may have numbers of 18, 27, 36, etc. By means of the alkaloid, colchicine, we are now able to double experimentally chromosome numbers in a wide variety of plants and produce $3n$, $4n$, $6n$, and $8n$ plants or sectors almost at will.

The Individuality of Chromosomes: Thus each species normally has a constant number of chromosomes, and the individual chromosomes have certain distinctive features such as position of centromere, relative length of arms, and secondary constrictions or satellites which give them characteristic size and shape. Actually, in most of the species best known cytologically it is possible to distinguish each chromosome of the haploid complement from the others on the basis of morphological differences.

In *Trillium*, for example, the haploid chromosome number is five. It is relatively easy to distinguish the five chromosome types, either in haploid or diploid tissue on the basis of size and shape (Warmke, 1937). There are three V-shaped chromosomes (median or submedian centromeres): a large V, an intermediate V, and a small V. The remaining chromosomes are a J-shaped chromosome, with one arm two or three times the length of the other, and a knobbed chromosome (sub-terminal centromere).

In maize all ten chromosomes are distinct and can be identified at the pollen grain division (McClintock, 1929) on the basis of length and position of centromere. More recently (McClintock, 1933), the chromosomes have been identified at pachytene; here the presence of prominent chromatic knobs on certain chromosomes, in addition to the other criteria, make identification more certain.

Very recently Satina, Bergner, and Blakeslee (1941) have shown that the twelve chromosomes in *Datura* are all morphologically distinct. Seven of the twelve in this case were found to bear satellites. These workers have been able to identify the extra chromosome in the primary ($2n + 1$ types), and what is even more striking, they have been able to identify the secondaries. The 9·10 chromosome, for example, bears a satellite on the 10 arm. The 9·9 secondary is equal-armed, and bears no satellite on either arm; the other secondary, 10·10, is equal-armed and bears satellites on both arms, as one would expect.

I think that gives a brief picture of the external structure of the chromosome; I shall now turn our patient over to Drs. Nebel and Huskins who will proceed to operate and reveal some of its internal structures.

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DISCUSSION

DEMEREK: It is generally thought that chromomeres correspond to genetic loci. There is good evidence for this in *Drosophila*. Does anyone in the audience know the present status of the evidence in plants?

McCLINTOCK: In maize, several color mutants may be referred to one chromomere of a mid-prophase meiotic chromosome. The chromomere in that plant may possess more than one gene.

DEMEREK: Belling thought the chromomeres in lilies corresponded to genes since the number he observed was comparable to that expected for genes.

HUSKINS: I am interested in the prophase drawing by Flemming, shown by Dr. Warmke, since loose chromosome ends are visible. This is of interest in connection with the erroneous concept of the continuous spireme, which apparently had its inception in a diagram by Flemming of a continuous thread.

WARMKE: Of course the paraffin section technique was in use at this time, and I rather think that Flemming's figure was drawn from sectioned material; in that case what appears to be free ends may have been cut ends. The drawing in question was taken from Wilson, 1928, "The Cell in Development and Heredity," page 126, and was labeled "Endosperm of *Fritillaria*."

DEMEREK: A question which has been raised in discussions of the physical aspects of chromosomes is, how far can a chromosome be stretched? The secondary constriction in *Drosophila* may give good material on this point.

KAUFMANN: In some *Drosophila* nuclei, smeared without great pressure, the chromosome may be so stretched that the piece distal to the pronounced secondary constriction in the left limb of the second chromosome may be on one side of the nucleus, the rest of the chromosome on the other side, with the continuum not visible. In other stages of mitosis, or from the evidence obtained from the salivary chromosomes, it seems that a thread of euchromatin connects the dislocated parts. In 2L then, a small

piece of chromonema can be stretched across the diameter of the nucleus.

DEMEREK: What is the factor of stretching?

KAUFMANN: Adjacent chromosome regions may be separated by as much as six micra.

GATES: It is possible that increase in chromosome length is a function of uncoiling of the chromosome spiral. Could Dr. Kaufmann's case be explained in this way?

SCHRADER: It should be pointed out that in the material of Pollister, referred to in Dr. Warmke's paper, the relation between the number of centrioles and akinetic chromosomes is not approximate but very exact.

WRINCH: What evidence is there as to the moment of duplication of the chromonema? Isn't this the time when the chromatin is not visible?

WARMKE: This is a question I should prefer to postpone until later in the Symposium. Both Dr. Nebel and Dr. Huskins I am sure will have something to say about this in connection with chromonemata, and there may also be evidence from X-ray breaks in still other papers.

JAKUS: What relation might the granule in the kinetochore have to spinning out of the mantle or traction fiber?

WARMKE: I am not able to answer this question.

HUSKINS: It should be realized that the granule and the kinetochore may be separate entities.

WARMKE: Yes, that is certain. The primary constriction is a region of the chromosome in which spiralization does not occur. The centromere, where visible, is merely a tiny dark-staining sphere in the chromatic thread.

SCHRADER: The granule and the rest of the kinetochore are not indistinguishable. For the present the granule does not matter except for pure cytologists. Cytological and physiological study of the chromosomes may show its exact role later.

The first step in separation of the chromatids is not taken at the kinetochore in the forms with which I am familiar, despite the figure of Haney, which Dr. Warmke showed. The kinetochore is not necessary in all cases for separation.

WARMKE: The idea that anaphase separation is initiated by splitting of the centromere, of course is chiefly that of Darlington. I realize that there is much evidence against this; namely the demonstration that the centromere in some species at least may already be split in late prophase or metaphase and also the evidence of Carlson on acentric chromatids.

SCHRADER: Darlington cannot be pinned down to this, since he shows other figures where splitting does not begin at the centromere.

METZ: In some cases, as *Allium*, the kinetochore may not be split when separation begins.

WARMKE: Colchicine treated material supports Dr. Metz' statement; here the centromere region may remain attached while the chromatid ends are widely separated. Generally in normal material, however, it seems that anaphase separation starts in

the centric region and proceeds out the arms, with the ends of the chromatids being the last to part, regardless of whether or not the ends may have appeared to be free earlier.

METZ: I was not discussing anaphase movement but primary splitting. The chromatids may separate at metaphase or during metaphase, before anaphase separation occurs.

SCHRADER: Two steps are involved. The kinetochore undoubtedly leads the way after the first step has occurred.

BERGER: What does the salivary gland chromosome show about the length of the uncoiled chromonema—does it represent the length of the completely uncoiled chromonema of the resting nucleus? I think the salivary gland chromosome is mistakenly called a prophase stage, but is really a resting stage, and so may be completely uncoiled.

METZ: We do not know anything about this. Most people think the salivary gland chromosome is longer than the uncoiled chromonema, but we cannot tell.

BERGER: Since the resting nucleus chromosome is too small to see, the salivary chromosome gives the only observable material on some points.

DEMEREK: The length of the salivary chromosome was discussed several years ago by C. B. Bridges, Astbury, Wrinch and others; the salivary chromosome is about 100 times as long as the metaphase chromosome, and about 10 times longer than could be accounted for by metaphase uncoiling.

SCHULTZ: Is such a comparison legitimate? Since the size of the metaphase chromosomes themselves differs in the different cells of the organism, we do not know what size of the coiled chromonema to use as a base line.

GATES: Manton, working with *Osmunda*, measured the chromonema at different stages of meiosis and found that apparent changes in length are the

result of spiralization and despiralization, from the evidence of actual measurements.

MULLER: This is related to the question of whether the genes lie directly against each other or are discontinuous and separated by other material. If they are not separated, then differences in length of the chromonema must be due to uncoiling of some kind.

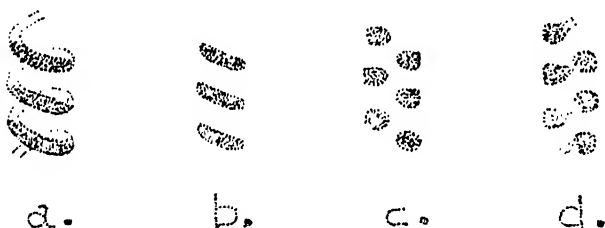
SCHULTZ: This is what I had in mind—the question of the formation of new material in the chromosome, rather than simple uncoiling at telophase.

PIZA: *Tityus bahiensis* Perty, a Scorpion belonging to the family Buthidae, very common in Brazil, has six diploid chromosomes which are provided with two terminal centromeres each. The chromosomes of the spermatogonia are curved or sinuous. At metaphase, because their two ends of chromosomes are in the plane of the equator, their entire body is forced to lie in the same plane. At metaphase of the first meiotic division, the three bivalents, which are rod-shaped, very often form a triangle. The lateral views of these chromosomes show that they are perfectly separated, having the ends turned towards the poles, to which they are connected by spindle fibers. At anaphase the chromosomes of each pair, as they are going to the poles, assume the shape of an arch hanging from the poles by the fibers inserted at its ends. At the second division of the spermatocytes the chromosomes are smaller and thinner, but they show the same general behavior. At anaphase they look like minute parachutes falling down from the equator to the poles. Due to the localization of the centromeres at the extremities, the fragments originating from spontaneous breakages behave like ordinary telomitic chromosomes, and are able to mate with the corresponding parts of the unbroken partner. (Piza; SCIENTIA GENETICA 1:255-261, 1939.)

STRUCTURE OF TRADESCANTIA AND TRILLIUM CHROMOSOMES WITH PARTICULAR EMPHASIS ON NUMBER OF CHROMONEMATA

B. R. NEBEL¹

In using the microscope on objects of a size ranging close to and below the resolving power of visible light optics, a number of rules must be borne in mind before the images observed or photographed can be translated into three-dimensional models. Two adjacent dark bodies or spots closer than $1/4$ of a micron will not be separated. (The theoretically possible slightly higher resolution appears not to be of practical importance in the present material.) A spot or a thread with a diameter less than $1/4$ of a micron will not be seen at all or will appear as a very vague shadow. Thus apparent discontinuities of structure may be caused by attenuation of a structure actually continuous. Fictitious images may be caused by refraction and by interference. The human eye distinguishes macroscopically between absorption and refraction by means of experience. At dimensions below one micron this experience must first be built up.

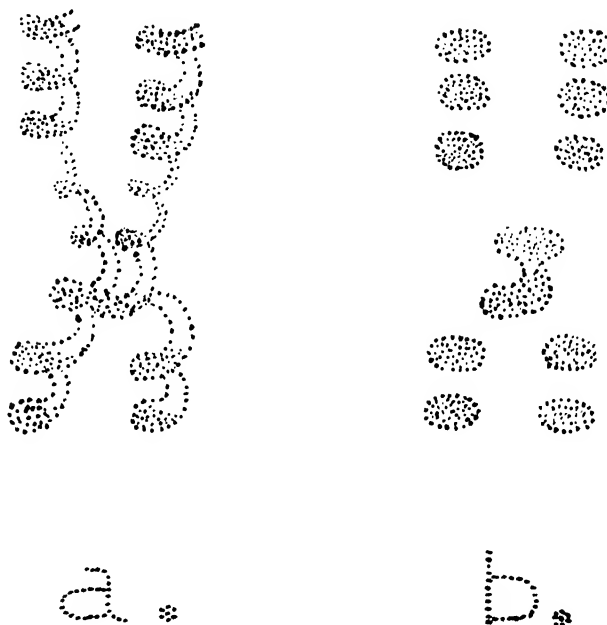


TEXT FIG. 1. Drawing of a simple spiral, diameter of gyres approximately $1/2$ micron; *a*, the object proper; *b*, *c*, and *d* microscopic images of this spiral; *b*, upper focus; *c*, middle focus; *d*, lower focus.

Text Figure 1 shows a spiral and three successive optical sections through this spiral, the gyre of which is more than a micron in diameter and the lumen of which is more than half a micron across. The first two sections, *b* and *c*, conform to expectation but the lowest focus, *d*, does not give a concise image of the lowest level of the gyres. The overlying upper levels of the spiral interfere in such a way as to blur and render indistinct the lowest level.

In Text Figures 1*a* and 2 the assumption is that the spirals under observation are semi-opaque to the light from the condenser.

In Text Figure 2 it is assumed that the spirals under observation (*a*) are partly transparent, more



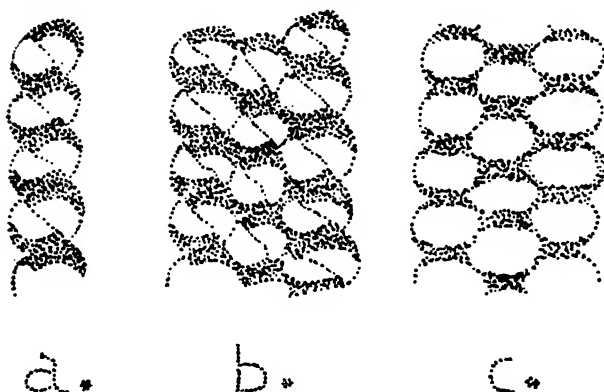
TEXT FIG. 2. Drawing of two spirals, diameter of gyres approximately $1/4$ micron; *a*, the object proper; *b*, microscopic image of same at all critical foci; details are lost by limit of resolution and through refraction.

highly refractive than their surroundings and of a diameter below $1/4$ micron. Thus, only those parts of the spiral can be seen where two parts of the thread combine or overlap. Where the spiral is attenuated even two thicknesses will not make it visible. Thus in *b*, showing the assumed image of *a*, the spiral is broken up into discontinuous dots, which upon focusing will follow a short distance but not give a continuous image. The distance between the two turns in the center is below the resolving limit so that the two spirals give only a single image. Just above this spot no image at all is obtained because the individual threads are too thin.

Text Figure 3 assumes that a transparent spiral (*a*) is under observation, which is more highly refractive than its surroundings. In *b* three such spirals have been set side by side closely. In *c* the microscopic image of such a system is drawn assuming each spiral to be about $1/2$ micron thick. Text Figures 3*a*, *b* and *c* should be compared also with Figures 7 and 8 which illustrate the seriation of light and dark areas from glass spirals viewed in transmitted light. Text Figure 3*c* illustrates how

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an image of this type will show series of wavy light and dark bands running at a pitch of about 35 degrees across the face of the figure.



TEXT FIG. 3. Schematic images of glass spirals viewed in transmitted light; *a*, a single tightly wound spiral; *b*, three spirals side by side; *c*, the same image as in *b* but more simplified.

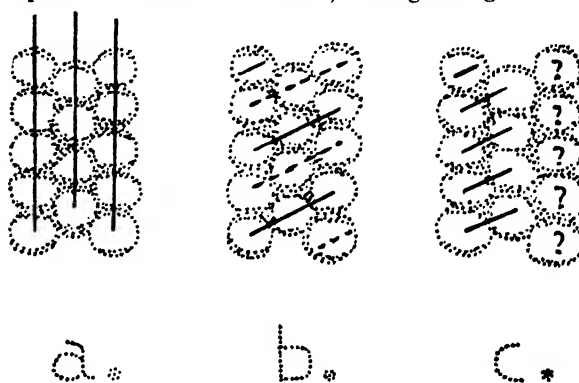
In Text Figure 5 *a* the upper gyres of a two threaded spiral are shown which exhibits a major and a minor coil. If each individual thread is less than 1/4 micron thick, threads can be seen only where overlaps increase the visible thickness, and at the edges, where the major coil turns away from or towards the observer. Text Figure 5 *b* shows the microscopic image of such a system of spirals; in the center a light area is caused by the fact that this structure as a whole gives a "glass rod effect," showing dark margins and a bright central line. The outermost marginal row of black dotted areas on either side corresponds to the turning points of the major coils. The two rows of dotted areas closer to the axis, one on either side, correspond to the first turns of the minor coils, counting inward from the

margin on the upper gyres. It is assumed that the entire structure of Text Figure 5 is not thick enough to allow a clear image to be formed of the lower level of the major coils.

Microscopic images corresponding to Text Figures 1, 2, 3 and 5 will be shown in photographs (Plates I and II), in some of which a paired replica has been touched with ink to emphasize the contrast between the light and dark areas of the photograph. On the correct assignment of the photographs to a corresponding schematic spiral type depends the correct interpretation of chromonema number and behavior.

MEIOTIC CHROMONEMATA

Figures 1, 2, 5 and 6 illustrates spiral types which may be assigned to the pattern of Text Figure 1. In Figures 1 and 5 the upper focus of the major spiral of meiosis is shown, transgressing the full



TEXT FIG. 4. Three interpretations of the same pattern in terms of transparent refractive spirals; *a*, axes of spirals vertical, side by side; *b*, axes of spirals diagonal; *c*, if a single gyre of a vertical spiral should give two rows of light spots, the third row "(?)" not accompanied by a fourth is unaccounted for.

FIGURE LEGENDS FOR PLATE I (see facing insert)

FIGS. 1 and 2. *Trillium grandiflorum* first meiotic anaphase chromatids; 1 upper, 2 lower focus, showing tertiary split and minor coil; the lower focus does not give a clear image of the lower level of the gyres; mark the diameter of the minor spiral as shown near arrow in 1.

FIGS. 3A and 3B. Late first anaphase in *Trillium*; upper focus showing light and dark dots in checker board arrangement in two chromosomes; 3A untouched; 3B the dark areas have been blackened and dotted with india ink.

FIGS. 4A and 4B. Late first anaphase in *Trillium* in medium focus; 4A individual chromonemata appear to give separate images in area indicated by arrow; 4B the same picture touched with india ink to mark apparent position of individual chromonemata in favorable area.

FIGS. 5 and 6. *Tradescantia reflexa* absorption images; 5 prometaphase of meiosis coils stretched with half normal Ringer at pH 7.6 showing minor coil and secondary split; 6 first anaphase showing tertiary split at turning points of gyres and at upper end of left chromatid.

FIGS. 7 and 8. Glass models photographed in transmitted light; 7 single spiral closely coiled; 8 two single spirals side by side; touched with india ink to make dark areas plain; the central row of light spots represents interspaces between the two spiral columns.

FIGS. 9 and 10. Late somatic prophase in *Trillium* showing four chromonemata at end and transgressing the kinetochore respectively. (Slides loaned by Dr. L. W. Sharp.)

FIGS. 11 and 12. Glass spirals photographed head on in transmitted light; 11 single spiral snugly coiled transmits light to end; 12 two spirals loosely coiled transmit little if any light to ends.

FIGS. 13 and 14. *Tradescantia reflexa*, microspore divisions; 13 metaphase in end view, showing 4 light areas in right hand partner; 14 telophase showing chromosome ends at different angles with reference to the observer, marked by simple and feathered arrows. (Compare figs. 11 and 12 with 13 and 14.)



PLATE I (see facing text page for legends)



15A.



15B.



15C.



16A.



16B.



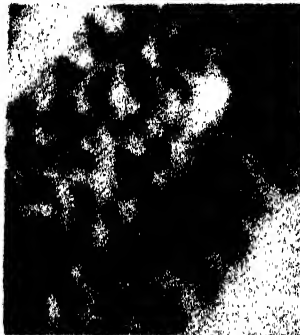
17.



18.



20.



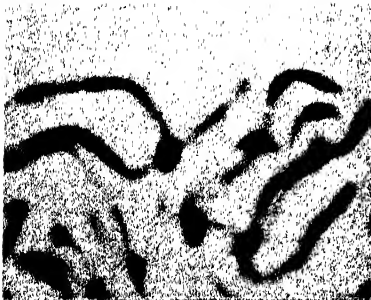
21.



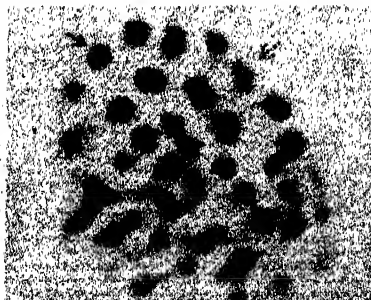
19A.



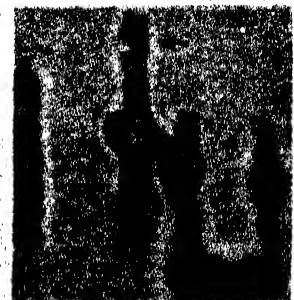
19B.



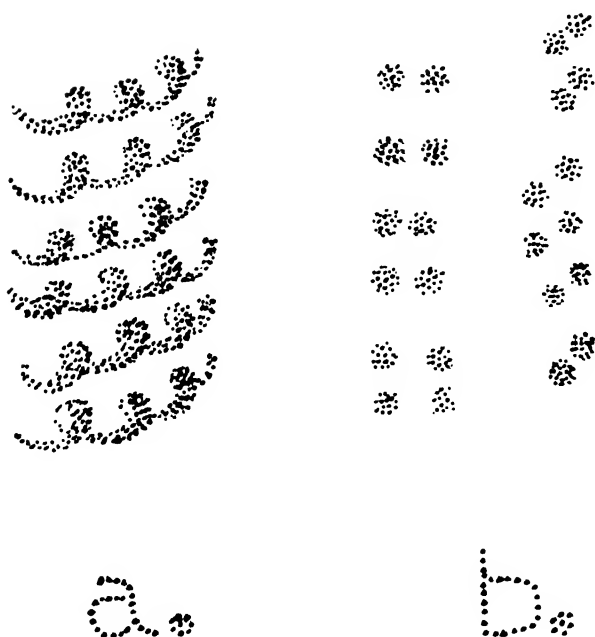
22.



23.



24.



TEXT FIG. 5. Drawing of the upper level of two stranded spiral with major and minor coil; *a*, physical image; *b*, microscopic image, with central area deleted due to "glass rod effect."

width of the chromosome. In Figures 2 and 6 the lower medium focus of this type of spiral is shown, giving the turning points as dots and the lower level of the gyres somewhat indistinctly.

A casual inspection of Figure 1 (the distal three gyres of the major spiral are under discussion) seems to show a light median line. This suggests two separate vertical columns of two stranded spirals. The lightening of the median line is due to the

"glass rod effect" of the entire chromosome. Careful visual observation shows the spiral to transgress the entire width of the chromatid involved.

What is the optically visible thread number in the first reduction division in *Tradescantia* and *Trillium*? Figure 5 shows only the two chromatids for a prometaphase chromosome; this photograph also shows the minor coil which will be discussed below. Figure 1 shows two threads in the end of a half dyad at first anaphase in *Trillium* and Figure 6 shows half a dyad with its mate in first anaphase of *Tradescantia*. In Figure 6 the doubleness is clear at the end and at several turning points, while in Figure 1 the doubleness is suggested by the contour of the turning points. In *Trillium* and *Tradescantia* first meiotic anaphase reveals the presence of half chromatids, that is, the tertiary split is visible, and the dyad is fourpartite.

With further progress of nuclear division, the first telophase in *Trillium* yields the images shown in Figures 3A and 3B, 4A and 4B. The upper focus no longer shows a continuous band across the width of the chromosome but instead a checkerboard or honeycomb pattern is seen. This type of image may correspond to the Text-Figure 3*b* and *c*. Two spirals with wide gyres have been replaced by four parallel non-coaxial spirals with narrow gyres. This conclusion is made more likely by the lower focus image shown in Figures 4A and 4B which seem to show four more or less parallel irregularly coiled strands running through the length of the chromosome. What appears as four more opaque or thicker regions of the chromonemata has been marked with ink in Figure 4B. If this interpretation is correct the dyad is now eight-partite showing the quarternary split. This interpretation requires that an individual thread contracts in length between anaphase and telophase approximately by a factor of two

FIGURE LEGENDS FOR PLATE II (see facing insert)

FIGS. 15A, 15B, and 15C. Somatic telophase chromosome of *Crocus* sp. showing the honeycomb pattern at different focal levels; 15A untouched photograph; 15B dark areas of photograph accentuated with ink; 15C the same at different focal level.

FIGS. 16A and 16B. Telophasic somatic chromosome fragment; 16A untouched; 16B dark areas accentuated with ink; four chromonemata in parallel or two chromonemata plectonemically coiled with a minor somatic coil superimposed on the "standard" somatic coil.

FIGS. 17 and 18. *Tradescantia* microspore division anaphase; 17 at least two spirals are indicated at the points marked by arrows; 18 the same; if two strands are present they may be para- or plectonemic; if only two strands are present they appear to show a minor somatic coil.

FIGS. 19A and 19B. *Tradescantia* meiosis prometaphase pretreated with $\frac{1}{2}$ normal Ringer pH 7.6; 19A shows checkerboard or ratchet arrangement of dark spots indicating the presence of the minor meiotic coil; 19B dark areas emphasized by spotting with ink. The two strands involved are considered paranemic.

FIGS. 20 and 21. Pachytene and first anaphase of *Trillium*;

20 the individual pachytene threads appear optically single; spacing between successive dark areas $\pm \frac{1}{4}$ micron; 21 anaphase figure juxtaposed to show that here also successive dark areas are approximately $\frac{1}{4}$ micron apart.

FIGS. 22, 23 and 24. Somatic prophase and telophase of *Amblystoma* sp. (slide loaned by Dr. C. L. Parmenter) and first meiotic anaphase in *Melanoplus* sp.; 22, no subdivision of chromatids appears possible; 23, differentiation into chromatids appears obvious; 24, no subdivision of chromatids appears visible; since the visible coils in *Melanoplus* are about as large as the minor meiotic coils in *Tradescantia*, coiling in *Melanoplus* is either relatively more coarse or, if it contains the same details as *Tradescantia*, these are below the range of optical resolution.

Magnifications

FIGS. 7, 8, 11, 12	x 1
FIGS. 22, 23, 24	x 2000
FIGS. 1, 2, 3, 4, 5, 6, 9, 10, 13, 14, 17, 18	x 3000
FIGS. 15, 16, 19	x 3500
FIG. 20	x 4500
FIG. 21	x 5500

and increases in thickness from below the limit of resolution to above $1/4$ micron.

There are two other alternate explanations for the telophase images of Figures 3 and 4. If the minor coil of meiosis expands but slightly from its anaphase diameter and if the thickness of individual threads approximately doubles, then the honeycomb image of Figure 3 may be the result of the expanded minor coil without an increase in thread number. In this case the telophase of the first division in *Trillium* shows only the tertiary split. The foregoing possibilities of interpreting the first telophase chromatid of *Trillium* are diagrammatically represented in Text Figures 4*a* and *b*. One has the task of seriating three parallel rows of staggered spiral gyres. Text Figure *a* serializes the spots vertically, *b* serializes the same pattern in terms of a major and a minor coil, adjacent gyres running diagonally across the face of the pattern.

If Figures 3A and B are to be interpreted according to Text Figure 4*b* then Figures 4A and B conform to the scheme laid out in Text Figure 5*a* and *b*. The eight black ink areas entered in Figure 4B correspond to eight dotted areas at any one of the three levels shown in Text Figure 5*b*.

Finally there is a third possibility of seriation, mentioned for completeness rather than for its likelihood (Text Figure 4*c*). Figure 8 shows two glass spirals. In the second gyre from the top, two light spots are visible for a single turn in *each* spiral. According to this phenomenon the "triple ratchet" pattern could be seriated according to Text Figure 4*c*. This leaves one row of spots unaccounted for. There is another objection to this last interpretation. In Figure 8 the dark bridges which separate adjacent white spots of the same gyre are too narrow to promise optical resolution in terms of chromosomal dimensions.

SOMATIC CHROMONEMATA

Figures 9 and 10 are taken from *Trillium* slides loaned by Dr. L. W. Sharp. They are taken late in prophase and there can be no doubt that four strands in Figure 9 transgress the kinetochore or form part of the kinetic region in Figure 10.

If one wishes to investigate how far back the fourpartite condition extends in the somatic cycle it becomes necessary to use more indirect means of investigation. Previous studies have led to the conclusion that somatic telophase shows four chromonemata in *Trillium* and *Tradescantia*. This finding requires recapitulation. Chromosome ends have been used extensively to show that somatic chromosomes at telophase appear to contain four chromonemata. For comparison glass spirals were photographed in end view and Figure 11 shows that a snugly coiled spiral will transmit light whereas a loosely coiled one (two are shown in Figure 12) will not.

Figures 13 and 14 may be compared with Figures 11 and 12. Figure 13 is from a late metaphase in *Tradescantia* in the first division in the pollen grain,

and Figure 14 is from a corresponding telophase. Figure 13 shows four light dots in the upturned end of one chromosome. Figure 14 shows four dark end knobs in the chromosome marked by the feathered arrow and four terminal regions in the chromosome marked by a simple arrow; the latter chromosome faces the observer obliquely. Somatic telophases in side view present a problem similar to that encountered in meiotic telophase. A chromosome of *Crocus* is taken in Figures 15A and B, and, in slightly lower focus, in 15C, again showing the apparently typical triple-ratchet of light dots surrounded by relatively darker areas. If here again the alternate explanations of Text Figure 4*a*, *b* and *c* are to be applied, preference has previously been given to the first alternative, because the majority of telophase images appear to indicate longitudinal rather than transverse arrangement of chromonemata. If one wishes to consider the explanation of Text Figure 4*b* one would need to postulate a minor coil of mitosis originating at anaphase or earlier or one would need to postulate spiralization of the entire anaphase chromosome. For the latter mode there is no observational basis.

An absorption type image was recently found in *Tradescantia* pollen grain material from 75 r X-radiation. Figure 16 A and B show this telophase fragment which contains four chromomeric regions side by side in its lower half. Of these three are plainly visible in a single photograph with the fourth one partly showing under the first chromomeres on the side of the arrow. The upper end of this fragment also appears fourparted. It thus seems that the telophase chromosome in somatic divisions of *Tradescantia* at least may be fourpartite.

Figures 17 and 18 show early anaphase in side view. The regions marked by arrows in Figure 17 indicate the presence of two chromonemata, corresponding in part at least to the conditions illustrated in Text Figures 2*a* and *b*.

The "V"-shaped chromosome of Figure 18 shows a "triple-ratchet" distally in the long arm and a "double-ratchet" proximally. The chromosome marked with the three-feathered arrow is pictured only in extreme upper focus. It shows a "double-ratchet" in this focus and probably corresponds closely to its mate.

The photographs so far available of somatic anaphase chromosomes present extreme difficulties of interpretation. Simple cases, where straight bands transgress the width of the chromosome are the exception. The double and the "triple-ratchet" can be explained in two ways, according to Text Figures 1*c* and 4*a*, or 4*b*. The structure of the somatic anaphase chromosome in *Tradescantia* is thus still considered a puzzle. Certain images, such as Figure 16, suggest four chromonemata non-coaxially spiraled in paranemic arrangement. It is possible that early anaphase figures as shown in Figures 17 and 18 represent two paranemic coils engaged simultaneously in separating from each other and in

multiplying visibly. It is also possible that anaphase chromosomes in *Tradescantia* contain two coaxial plectonemic strands with a minor somatic coil running over the standard somatic helix. These plectonemic strands, if such is the arrangement, are believed to unravel by rotation towards late telophase and occasionally the strands may multiply from two to four. Finally it may be recorded that the number of chromomeres were counted in leptotene chromosomes of *Tradescantia*; this number ranged around 70. Within ten percent an equal number was found for the number of minor somatic coils in a single somatic anaphase chromatid, under the assumption that the anaphase chromosome consists of two coaxial spirals with the number of minor spirals averaging seven for each complete turn of the standard somatic coil. The meaning of the coincidence of these two numbers is not understood.² Also under the assumption of two coaxial coils with a standard mitotic and a minor mitotic helix the leptotene thread does not decrease its length in *Tradescantia* between leptotene and somatic anaphase. Leptotene chromosomes of *Tradescantia* subjected to the electron microscope showed indications of banding or periodic changes in density. This evidence must be confirmed before it is accepted. It appears too early to correlate at this time into a mechanical scheme the number of chromomeres at leptotene, the apparent number of minor somatic coils of anaphase and the relative length of the chromonemata at the two respective stages. At this time the accumulated evidence on somatic anaphase chromosomes in *Tradescantia* is still confusing with regard to details. Why it is confusing aside from secondary considerations is illustrated by inspection of the minor coil of meiosis as shown in Figures 19A and 19B. The minor meiotic coil in *Tradescantia* is a true spiral, not a wave, and from *a priori* and *a posteriori* considerations this structure must be paranemic. Are the two strands (chromatids) which are shown as a double-ratchet in Figures 19A and 19B coaxial or not? While the former interpretation is preferred, it is not cogent. Thus in meiosis one has the number of chromatids and their separation in first anaphase as anchor points and yet the optical interpretation is subject to argument. With regard to mitosis there are no anchor points as the number of chromonemata before and after anaphase is under dispute; also there is no obvious separation of chromatids after mitosis; thirdly the chromonemata are more closely packed in mitosis. With proper experimental methods, however, the last problem may be approached.

Figures 20 and 21 illustrate the respective size of pachytene coiling in *Trillium* and the minor coil or wave in first anaphase. In both cases the spacing between successive dark areas is about 1/4 micron.

² At meiosis the number of minor meiotic coils at metaphase falls to within the range of 50 per chromatid in *Tradescantia*.

Figures 22, 23, and 24 are included in this discussion to illustrate the aspect of chromonemata in other organisms. Figures 22 and 23 are from prophase and telophase of mitosis in *Amblystoma* sp. the slides being loaned by Dr. C. L. Parmenter. Here the stained unit is the chromatid with no subdivision discernible. In fact, chromatids are not even separate optically at all levels in the prophase figure, whereas this division is fairly obvious in telophase. There is no indication of coils in these photographs. Figure 24 shows first meiotic anaphase in *Melanoplus* sp. The tertiary split is not visible. The coils observed correspond in size to the minor meiotic coils in *Tradescantia*. The question of a smaller coil below the limit of visibility must remain a conjecture.

In the foregoing an interpretation of meiotic and mitotic chromonemata was attempted using a number of diagrams for guides. Due to limits of resolution the problem is considered unsolved. There is no stage which would make interpretation impossible, but due to the interplay between refraction, absorption and interference and, due to the possibility not only of a minor spiral persisting in meiosis but of a minor spiral existing also in anaphase and telophase of mitosis a final interpretation of all photographs cannot be reached at this time.

DISCUSSION AND SUMMARY

Two reviews of the literature were published by Nebel and by Kuwada in 1939. Both reviews agreed that the telophase chromosome in mitosis of *Trillium*, *Tradescantia* and plants with chromosomes of similar size is fourpartite, the meiotic tetrad eight partite. Abraham in 1939 and Coleman in 1940 consider the anaphase chromosome bipartite, prophase fourpartite. Both workers consider the spirals paranemic. MacKnight (1940) has suggested a new working hypothesis of coiling. Resende (1940) considers chromosomes double until prophase.

The work of Hughes-Schrader (1940) on coccids has been extended (see discussion) and reemphasizes the reality of the tertiary and occasionally even a quaternary split in meiosis.

Matsuura and Haga (1940) were able to obtain physically complete separation of meiotic half-chromatids through heat treatment. Kuwada, Shinke, and Nakazawa in 1939 rediscovered the coming together of somatic secondary sister chromonemata in prophase shown by Nebel in 1933. Kuwada and Nakamura (1940) explained certain induced changes in the appearance of prophasic and resting nuclei on the basis of four threads, secondary sisters showing a plectonemic coil. Iwata (1940) observed the tertiary split in meiosis of *Trillium*, this being more pronounced in the second than in the first reduction division. Aisima (1941) considers mitotic telophase fourpartite. His figure 16 *b* begs for an interpretation in terms of the viewpoints expressed in this paper. Here more than three dots appear across the face of somatic anaphase chromosomes.

If there is a minor and a standard somatic coil, such images are understandable.

In summary, in this paper it has been shown that the anaphase and telophase somatic chromosome of *Tradescantia* requires more study, the exact path and hence the number of chromonemata are not established for all images obtained. The author believes the telophase chromosome to be fourpartite at least at the distal ends regardless of the problem still ahead of ascertaining the exact path and number of the chromonemata in the main body of the chromosome, of which only indirect optical evidence can be obtained.

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DISCUSSION

COLE: In the last diagram shown, is *a* the result of three two-stranded spirals, which are close and the turns close-packed?

NEBEL: Yes.

COLE: What would the paranemic pattern look like?

NEBEL: This would be too difficult to make of glass, but if it were possible, the paranemic image would be much the same as the plectonemic.

HUSKINS: You have shown that chromosomes can give alternative images and that a double spiral glass model can give both images, but have you shown that a single spiral model made of a glass rod could give both?

NEBEL: No, but it can be demonstrated that non-coaxial spirals can give a triple ratchet image.

COLE: Plastics would be better to use for models.

SCHRADER: The fact that the ends of the chromatids are seen indicates that the coils are coaxial with the chromosomes. If not, the ends would go to one side, and the dots seen at the end would be gyres of the minor coils seen from the side.

NEBEL: Both types of images seem to occur, making it possible that both interpretations are correct, even in the same material.

HUSKINS: Could you give briefly other evidence bearing on the number of strands?

NEBEL: *Aisima* shows more than 4 dots at one level across the width of the chromosome.

SCHRADER: If you make the coils shift on each other, you get beyond all optical illusion and are absolutely certain; this happens in Mrs. Schrader's coccid material.

HUGHES-SCHRADER: In the meiosis of the male *Llaveiella* the metaphase chromosome is at least four-parted, the tetrad eight-parted. The separation of the elements is complete, involving sheath, matrix and chromonemata. The parts of the chromosome move poleward at different rates, making their number clear beyond possibility of optical illusion. Occasionally the chromosome is further similarly divided along the quarternary split, giving a four-parted anaphase chromosome.

SPARROW: The important point is the type of spiral structure in these elements. Are coils visible, and if so are they paranemic or plectonemic?

HUGHES-SCHRADER: The coils are seldom analyzable, but each element must contain at least one coiled chromonema. This is frequently demonstrable in late anaphase and telophase in the spermatid.

SWANSON: Heat treatment (40° C.) of *Tradescantia* buds can reduce the number of major coils in some cells while leaving the chromosomes completely uncoiled in others. When the number of major coils is reduced, these heat-shortened chromosomes show not only major and minor coils, but frequently a sub-minor coil. Under *normal* conditions, the sub-minor may not be present. Also in some cells at 40° C., the coiling system is relaxed showing that at least four threads are present in each chromosome, and very possibly eight threads. Also this relaxation of the coils reveals that the half-chromatids as well as the chromatids can separate freely from each other laterally. This argues in favor of a paranemic spiral.

HUSKINS: Has Schultz seen the spiral structure with Caspersson's technique?

SCHULTZ: Caspersson has made photographs of *Tradescantia* which look pretty much like the stained preparations. How much smaller must the material be to miss seeing the split?

NEBEL: A factor of .75 will answer.

SCHULTZ: Apparently the larger the chromosome, the more strands we see. Might not occurrence of the split be related to size of the chromosome?

DEMEREK: What did Nebel find with electron microscope?

NEBEL: The presence of bands in the leptotene chromosome is suggested, and also the occurrence of strands of smaller diameter than that of the leptotene chromosome.

THE COILING OF CHROMONEMATA

C. L. HUSKINS

The condensed chromosome of mitosis or meiosis contains within its limiting membrane or interface one or more coiled chromonemata. The number of strands it comprises has been discussed by the previous speaker (Nebel, 1941) and this phase of the problem will be touched upon only insofar as is essential for consideration of the structure of the coils. The half-chromosome or chromatid is the unit of genetic segregation, crossing-over, etc., and it also appears for the most part, though not always, to be the unit of coiling. Since the term chromonema implies only a thin strand and there are serious differences of opinion on the number of strands present, coiling will be discussed as far as possible in terms of chromatids or half-chromatids. When the two chromatids of a chromosome are separated at mitotic anaphase or the anaphase of the second division of meiosis they become chromosomes and the half-chromatids become chromatids.

The coils of chromonemata may assume various forms and one form at different stages must receive different names for the sake of clarity in discussion. The most thoroughly studied coils are those of meiosis. Here there is present a large-gyred helix and along its length either a small-gyred helix or a waviness. These are the "major" and "minor" coil respectively (Huskins and Smith, 1935). At certain stages chromosomes or chromatids (coiled in the same direction) are twisted about each other. Such a pair of strands has been termed a relational coil (Darlington, 1935). Mitotic chromosomes at metaphase or anaphase have, in the opinion of our group (Sparrow, 1941) and many others, a helical chromonema similar to the major coil of meiosis but usually of smaller diameter. This has variously been termed a minor, a standard, and a somatic coil. The latter term will be used here. The coil of the preceding anaphase whether mitotic or meiotic may persist to the prophase of the next division. It is then termed a "relic coil" (Darlington, 1935). A helix consisting of two strands twisted about each other so that they cannot be separated without uncoiling is termed a "plectonemic coil," while two helices which are not intertwined form a "paranemic coil" (Sparrow, Huskins and Wilson, 1941). A plectonemic coil becomes a relational coil when it is pulled out longitudinally without untwisting. A paranemic coil similarly pulled becomes (or, rather, remains) two parallel strands.

The ontogenetic relationship of these different types of coils is the problem of chief interest for descriptive cytology. The mechanism of their formation is a problem involving experimental cytology and physico-chemical methods. Their possible (or rather probable) significance for genetics is a third inter-

esting issue. It will be difficult to keep these three aspects distinct but they will be discussed separately wherever this can be done in order to avoid confusion of observation with hypothesis or interpretation.

First the coiled structure of meiosis from the descriptive point of view! Japanese cytologists, especially Kuwada, (1939, and earlier references therein) have maintained since about 1933 that there is both a major coil and a regular, small-gyred minor coil along the major gyres at first metaphase and anaphase in *Tradescantia*. This "coil within a coil" or "double-coiled" structure was apparently first seen by Professor Fujii in 1926. The clearest illustrations of it until the recent ones of Sax and Swanson shown at the last meeting of the American Association for the Advancement of Science are those of Oura (1936) (fig. 1). The existence of a regular minor coil was assured for many organisms by various workers on the basis of observations which appeared to me (Huskins, 1937) to be inadequate. In many cases the so-called "minor spirals" are obviously drawn-out major spirals. In others they appear to be optical illusions. Huskins and Smith (1935) show the former in bridge chromosomes of *Trillium*. The latter error was revealed in *Avena* prophase chromosomes. Here two synapsing threads show a beaded chromomeric structure where they are widely separated and give the optical illusion of a spiral when they approach each other (6 o'clock in fig. 2). Though *Trillium* is exceptionally good material for the analysis of the major coil and has been studied extensively by many methods in our laboratory, we have never been able to see a regular minor spiral in it such as has been shown in *Tradescantia*. A waviness of the coiled chromonema is the most ever found in normal material. In material held at high temperatures during meiosis the major coil is often absent and *then* a small-gyred coil may be present (fig. 3), but it seems rash to identify this with the "minor spiral." Figure 5 shows a waviness along the gyres of the major coil in an asynaptic p.m.c. of a rhizome kept at an abnormally high temperature during meiosis. Since various effects are produced by temperature and by keeping the rhizomes too dry, it is obvious that caution must be used in drawing conclusions from such abnormal material or from slides treated to produce artificial uncoiling etc. (see Kuwada, 1939). In *Trillium* each of the four chromatids forms a relatively independent coil. In *Tradescantia* the paired chromatids are often so closely associated that at one time the existence of more than two coils in a bivalent or one in a first anaphase chromosome was doubted (see Kuwada, 1927 for one of the first clear demonstrations of the

double major spiral at first anaphase). I therefore thought for some time (Huskins, 1937) that the regular-gyred minor coil of *Tradescantia* might be an optical illusion similar to that of Figure 2 (and other lantern slides), particularly as I was unable to see indisputable evidence of it either in published photomicrographs or in many demonstrations. The recent photographs of Sax and of Coleman and Hillary (in press) together with slides made by Dr. S. G. Smith in our department convince me now of its occurrence in *Tradescantia*. I would, however, emphasize that the apparently clearest evidences of the minor spiral are in part optical illusions. The true minor spiral of each chromatid (or half-chromatid) is of very small diameter (see the second chromosome from the edge at 12 o'clock in fig. 1). Where two chromatids together form one major coil, two minor coils may appear as one particularly clear minor coil (see outside chromosome at 12 o'clock in Figure 1—there are definitely two chromatids making up this apparently single minor coil). Figure 4 is presented to show even more clearly the longitudinally double nature of the major spiral in *Tradescantia* (see especially the chromosome at 6 o'clock); it thereby gives some indication of the small diameter a single minor spiral must have in preparations which emphasize the spiral rather than the double nature of the chromonema. The other more obvious sources of error in the interpretation of apparent spiral structure, pointed out above, also remain; it would be invidious to pick out for citation a few of the many instances in the literature.

Satisfactory evidence of a regular minor coil has not yet, in my opinion, been found in *Trillium*. Kuwada (1938) has pointed out that there is no clear distinction between a waviness such as we see and a regular minor coil and he thinks the issue unimportant. With this I am pleased to agree from the point of view of descriptive cytology, particularly as we (Huskins and Smith, 1935 and since) have shown the major coil to arise from a waviness (in contradistinction to the opinion of many others that it is a regular coil from its inception). More recently

we have found the same transition in the development of the somatic coil (Sparrow, 1941). There is, however, most probably a difference from the point of view of the mechanics of coiling—those who see a coil at the earliest stages mostly assume some torsion mechanism or the rotation of the ends. This will be discussed later. It will also be shown that there are significant differences between *Tradescantia* and *Trillium* in the timing of the spiralization cycle relative to that of the spindle mechanism and the meiotic process.

This difference is most apparent in the structure of the chromosomes during the second division. In *Tradescantia* there is an interkinesis during which the major gyres are considerably straightened out. Then during the second prophase the minor coil of the first division develops directly into a medium-sized helix which is the equivalent of the "somatic" coil. A minor coil has not been demonstrated along its gyres. In *Trillium erectum* the chromosomes pass relatively unchanged from the anaphase of the first division to the metaphase of the second. The second anaphase helix is almost as large-gyred as that of the first (Huskins and Smith, 1935; Wilson and Huskins, 1939). A waviness along its length is sometimes evident.

The mechanism of coil formation may be discussed on the basis of our observations of the developmental cycle in the meiotic and post meiotic divisions in *Trillium* shown in the following lantern slides. These being mostly already published are not reprinted here. (Figure 6 is a photomicrograph of spiral structure at p.m.c. metaphase in a mutant wheat.) During diakinesis a waviness first appears in each of the four chromatids. By metaphase this has become a spiral and at anaphase when the chiasmata are resolved and the chromatids are independent it is particularly clear. At second metaphase and anaphase the coils are, as mentioned, very similar to those of the first division. In the prophase of the first post-meiotic pollen-grain division, the major spiral of the two meiotic divisions is present as a relic coil which gradually straightens out (fig. 7).

FIG. 1. First metaphase of p.m.c. meiosis in *Tradescantia*, treated with KCN to bring out minor spiral structure. From original photomicrograph by Dr. G. Oura, kindly presented by Professor Kuwada with permission to publish. Note apparently single, wide gyred minor spiral at 12 o'clock and narrow double spiral in chromosomes immediately below it and at 6 o'clock.

FIG. 2. Zygo-pachytene in *Avena sativa*. Note optical illusion of spiral in synapsed strands at 6 o'clock, 900 \times .

FIG. 3. Anaphase in *Trillium erectum* undergoing meiosis at high temperature, 800 \times (photomicrograph by Dr. G. B. Wilson).

FIG. 4. First metaphase in *Tradescantia reflexa*; prepared by Dr. S. G. Smith to emphasize double nature of major coil and to indicate the limiting diameter of a single minor coil. 1100 \times .

FIG. 5. First p.m.c. division of asynaptic *T. erectum*. Photomicrograph by Dr. A. W. S. Hunter. Note waviness along major spiral gyres, especially at 12 o'clock.

FIG. 6. First p.m.c. metaphase in a mutant 41-chromosome wheat, *Triticum vulgare*, 1200 \times . Photomicrograph by Dr. R. M. Love.

FIGS. 7-9. Prophase, metaphase and anaphase in first microspore division of *Trillium grandiflorum* (fig. 7 900 \times , figs. 8 and 9 1000 \times). Photomicrographs by Dr. A. H. Sparrow.

FIGS. 10 and 11. Early and late prophase of second microspore division in pollen tube of *T. erectum* 1100 \times and 700 \times . Photomicrographs by Dr. A. H. Sparrow and Isobel Hutcheson.

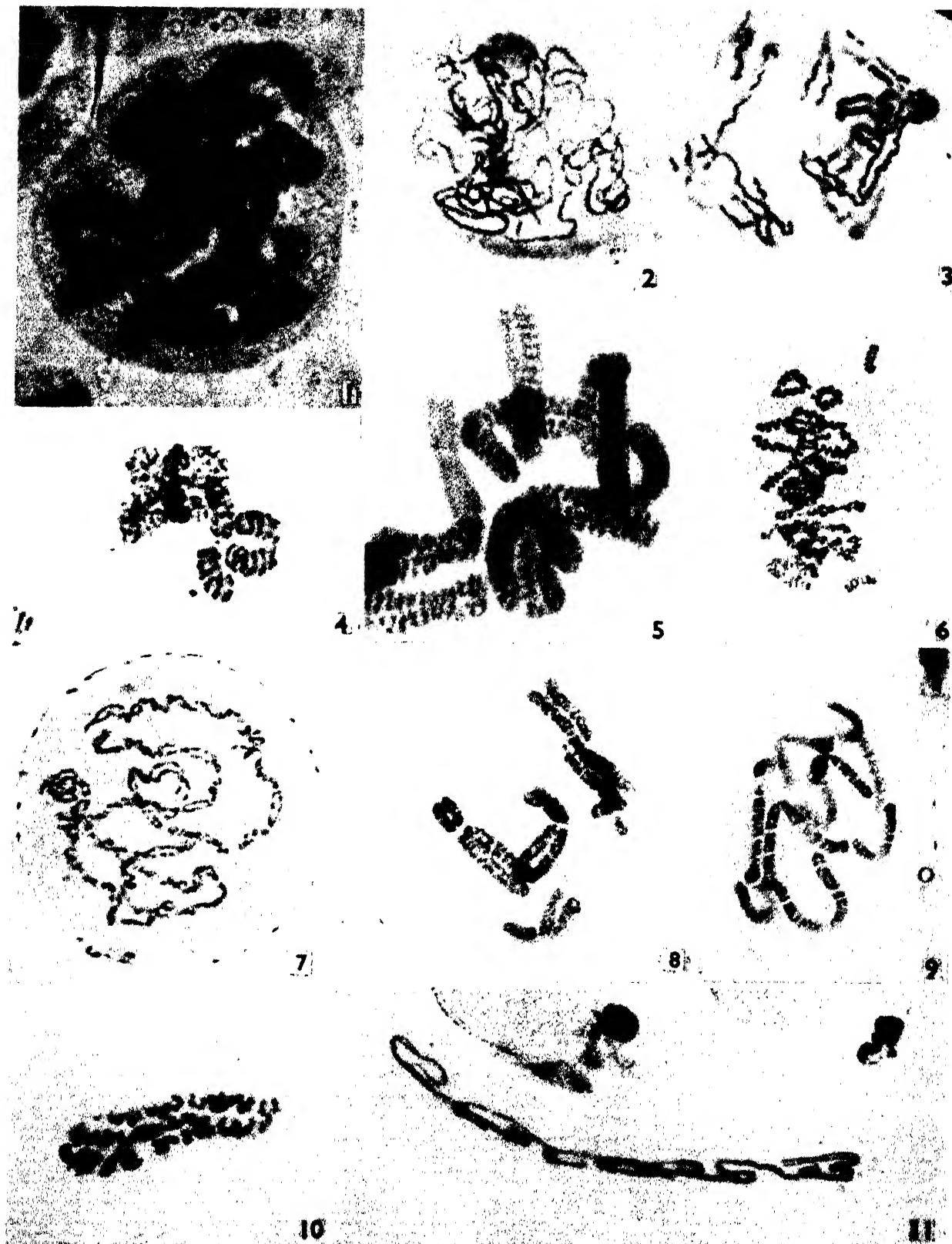


PLATE I (see facing text page for legends)

As it does so it is clearly revealed to be a double-stranded plectonemic coil (Sparrow, Huskins and Wilson, 1941). These intertwined strands gradually untwist and come to be parallel. A waviness is apparent along each of them and this develops by metaphase (fig. 8) into a helix like that of the major coil of meiosis, but with more numerous and closely packed gyres having fewer reversals. The gyre diameter is similar but more variable. There are about twice as many gyres per chromatid. There is by anaphase (fig. 9) fairly clear evidence of a minor waviness along it. In the second post-meiotic division, occurring in the pollen-tube, exactly the same process seems to occur, though it has not yet been analyzed as thoroughly (figs. 10 and 11).

Throughout the spiralization cycle changes are occurring in the length of the chromonema. To what extent these are true elongations and contractions of the material constituting the chromonema and to what extent they are due to the formation of and stretching out of a waviness or a minor coil with a diameter near the limits of microscopic resolution cannot be determined by present methods. Both types of change seem to be involved. The data from our laboratory on length changes in *Trillium* are presented in detail by Wilson and Huskins (1939), Sparrow, Huskins and Wilson (1941) and Sparrow (1941). In brief, the length of a complement of five chromonemata after acetocarmine fixation is approximately as follows. From a leptotene length of 920 μ it elongates by zygotene to about 1040 μ . By pachytene it has contracted to 640 μ and by the end of diplotene to less than 200 μ . By early diakinesis it is little more than 100 μ and by the end of diakinesis it is again nearly 200 μ . At metaphase its length is over 300 μ and by late anaphase it is about 350 μ . Throughout the second division it apparently changes little. Our data on lengths during the microspore division are limited and from two species which differ somewhat, but it appears that at prophase the chromonema is again about 1000 μ as at lepto-zygotene and that it shortens to about 650 μ at metaphase and lengthens to nearly 1000 again by anaphase.

During diakinesis, metaphase, and anaphase there is a "pellicle" (or "matrix" and "sheath") surrounding the coiled chromonemata and this, the "condensed chromosome" of general terminology, also changes in length, but in *Trillium* to only a very limited extent. Acetocarmine "chromosome length" increases from 86 μ to 125 μ during diakinesis, decreases to about 100 μ by metaphase, and may or may not increase again slightly during anaphase. At second anaphase it is about 80 μ . At the microspore metaphase it is slightly longer.

In *Tradescantia* the cycle is evidently different. Chromonema measurements have not, apparently, been made at many stages but it has clearly been shown by Sax and Humphrey (1934) that the chromosome contracts during the formation of the major coil in the first metaphase and anaphase.

The elongation between leptotene and pachytene has been confirmed by several workers on a number of organisms since it was first recorded by Gelei (1922) and Belling (1928). In various organisms metaphase chromosome length has been found to range between $\frac{1}{4}$ and $\frac{1}{8}$ of that at pachytene. The former ratio was found by Koller (1936) in two genera of Marsupials and the latter by Manton (1939) in the fern *Osmunda*. Smith (1941) found a ratio of about 1:20 in oocytes of *Diprion* (Hymenoptera). Our ratio of $\frac{1}{10}$ for *Trillium* is near that found by Sax and Sax (1935) for a number of plants. The outstanding question is whether or not this amount of "contraction" can all be accounted for by spiralization. If leptotene and pachytene chromonemata are already spiralled as a number of cytologists believe (they correlatively argue that chromomeres are optical or fixation artifacts produced in a small-gyred spiral) the assumption of one additional spiral would hardly be adequate to explain it. If they are uncoiled strands (and chromomeres a reality) as I believe, then the assumption of both a major spiral and either a minor coil or waviness could account for most, if not all, of the observed contraction. Uncoiled strands would also presumably be best adapted to the two by two pairing that occurs at zygotene.

The elongation of the chromonema which we have described during the formation of the major coil in *Trillium* has not yet been checked adequately in other organisms. It is obvious from illustrations that it occurs in many but to varying extents.

Contraction is generally less between early prophase and metaphase in mitotic than in meiotic divisions. Sax and Sax (1935) report an average reduction to $\frac{1}{3}$ in root tips and to about $\frac{1}{5}$ in microspore divisions. Our *Trillium* microspore measurements show a slightly greater reduction. In *Aggregata Ebheri*, Belar (1926) reported contraction varying from 2.5:1 to 33:1. Berger (1938) found the compound chromosomes in the ileum of *Culex* to be 10-15 times as long at mid prophase as at metaphase. *Drosophila* salivaries are, of course, about 70-100 times the length of ordinary metaphase chromosomes. This difference, at least, is not entirely due to spiralization, but it does not enter into our present problem since the elongation is presumably irreversible.

Studies of the reversals of direction which occur in the major coil of *Trillium* have yielded data very significant for the testing of various hypotheses on the mechanics of spiralization. Until recently speculation far outran observation in this field. It has been variously suggested that the direction of coiling is constant, that is regularly reverses at the attachment but nowhere else, that homologues coil in opposite directions, and that they coil together. Actually reversals have now been seen in both the somatic and meiotic chromonemata of a wide range of organisms and they seem to occur anywhere along the chromosome. In the *Tradescantia* major coil there

are relatively few changes. In *Trillium* they are abundant: The attachment is a point of random change; so are chiasmata. There are in addition adventitious reversals of direction about which almost all that can be said at present is that their frequency is proportional to chromonema length or gyre number (Huskins and Wilson, 1938; Wilson and Hutcheson, 1941). The observations of Matsuura (1935 et seq; 1941) agree but he has unorthodox views on the relation of chiasmata to crossing-over which affect his interpretation with reference to the spiralization problem also. He finds the frequency of reversals to be proportional to length and ignores chiasmata. The latter are localized and far fewer in number in his species, *T. kamtschaticum*, than in *T. erectum*. They could therefore have much less effect in a statistical analysis of the causes of reversals. We have, however, noted (Sparrow, Huskins and Wilson, 1941) that Matsuura's illustrations show an increased frequency of reversals at anaphase in the localized regions where the chiasmata are earlier situated.

The foregoing discussion has been concerned chiefly with structures of such a size that errors of observation are probably few and unimportant. The developmental relationships in the spiralization cycle now to be considered involve the interpretation of structures nearer the limit of microscopic resolution. There are therefore sharper differences of opinion to be taken into account. Darlington's (1935) theories of spiralization assume, as do all of his other theories, that "splitting" of the somatic chromosome occurs in the resting stage immediately preceding the division in which separation occurs of the chromosomes then constituted. He cannot therefore consider half-chromatid spirals or the occurrence of a metaphase or anaphase "split" the plane of which might determine the formation of a plectonemic spiral and a relational coil. Nebel, on the other hand, sees up to four strands in a somatic anaphase chromosome. During meiosis the leptotene strands are single according to Darlington and the split which produces the tetrad of chromatids occurs during pachytene. No further split occurs until the resting stage after the second division. Most other cytologists now believe that the leptotene singleness is only apparent, and many see a "tertiary" split becoming visible during diakinesis. It is clear in *T. erectum* and in some cases we can observe the relationship of the half-chromatids, which is plectonemic. In *T. grandiflorum* it is particularly clear at first anaphase (Sparrow, Huskins and Wilson, 1941). In desynaptic *Trillium* the half-chromatids are widely separated, but one must, of course, be cautious in arguing from such material. The illustrations of those who see two chromonemata at somatic anaphase nearly all indicate a plectonemic relationship. Further, Darlington (1935) and Upcott (1938), amongst others, show a plectonemic relic coil in the microspore prophase, so that there is observational agreement on the point essential for

the present issue. There remains the difference in interpretation: Darlington considers somatic prophase relational coiling to be due to an active twisting; we see no evidence of twisting and consider that the relational coil is a carry-over from the previous division in which the half-chromatids are plectonemic (Sparrow, Huskins and Wilson, 1941; Sparrow, 1941).

This concept leads to a dualism. The chromatid major spirals of meiosis are paranemic in *Trillium*. Only very rarely have they been reported as intertwined (see Matsuura, 1940). Shinke (in Kuwada, 1938) has clearly shown interlocked gyres in *Tradescantia* artificially uncoiled by ammonia. Kuwada argues that this can occur in *Tradescantia* because there is an interphase during which the chromatids can become untwisted, but that in *Trillium*, which lacks a meiotic interphase, "such a case would not be expected to occur." It should, however, be realized that if these two chromatid major coils of *Tradescantia* were normally paranemic, as in *Trillium*, and the treatment caused uncoiling by rotation, that would account for the intertwining. In any case, sister chromatid major coils are ordinarily paranemic. This accords with our concept of the mechanism of their formation. They result we believe from extension of the chromonema within a confined space, the pellicle, and not from active twisting. They are independent strands when formation of the major coil begins, and in *Trillium* at least, chiasmata reduce or prevent rotation relative to each other during coiling. The dualism has thus, according to our observations, an obvious explanation. The problem must, however, be carried further back to the time of reproduction of the meiotic chromosomes when the chromatids were formed.

A more serious dualism remains, but it is possible that it may rest on inadequate observations: at diplotene homologous chromosomes are often twisted about each other—hence the once common term "strepsitene." It is commonly believed now that zygotene and pachytene chromosomes are similarly relationally coiled. Darlington (1935) concluded this from McClintock's (1933) striking photomicrographs of maize. She has not confirmed this interpretation. We think that in wheat and oats which, like maize, have very clear pachytene chromosomes, many of the apparent twists are merely overlaps (fig. 31). The issue is a difficult one turning upon the interpretation of optical levels of strands near the limit of resolution. If, as Darlington assumes, the pachytene strands are relationally coiled through an active twisting, due to an internal, molecular torsion, and that the diplotene twists are the residuum after crossing-over (itself caused by the torsion) has reduced the number, then the chromosome relational coils of meiosis and the chromatid relational coils of mitosis appear to arise from two very different mechanisms. It seems, however, to be within the bounds of possibility that the meiotic coiling process may be the reverse of that postulated by Darling-

ton. It may be that zygotene chromosomes are less intertwined than diplotene, and that diplotene relational coils may be caused, not eliminated, by chiasmata. If so, molecular pattern may directly, not indirectly through torsion, determine or influence crossing-over. But at this point we enter the purely speculative, and I must remember that we are trying to be as nearly inductive in our research as is possible in a field in which, admittedly, little or nothing can be seen if one does not know what to look for.

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DISCUSSION

DELBRÜCK: Is there consistency in the direction of coiling in different divisions?

NEBEL: Variability does exist from cell to cell, and hence randomness of coiling or near-randomness exists.

SCHULTZ: It is usually thought that lengthening is due to despiralization. But cannot loss of material in the chromosome occur during the spiralization? Do we know whether there is a constant amount of material in the chromosome?

HUSKINS: We know there is not.

SCHULTZ: Then exchanges of material, synthesis and breakdown must occur in lengthening and in spiralization.

HUSKINS: This cannot be determined by any means so far used.

WRINCH: How far must the chromonema be uncoiled for duplication to occur?

HUSKINS: This must be variable, since there is so much variability in coiling between different forms.

WRINCH: What occurs during the time when you cannot see the chromosomes?

NEBEL: This is answered by the findings of Sparrow, Huskins and Wilson.

SPARROW: There is good evidence that the relic coil in microspore prophase is the same coil as that of meiosis, since the number of reversals of direction of coiling is the same.

WRINCH: Is there a torsion effect in the mechanism of spiralization?

HUSKINS: Torsion directed from one point is ruled out, and the reversal of direction probably rules out all torsion mechanisms. But the molecular pattern may influence coiling.

WARMKE: If the chromosomes are elongating from diakinesis to metaphase, how is spiralization which occurs at this period accomplished?

HUSKINS: It is the chromonema that elongates, not the chromosome.

JAKUS: What has Dr. Huskins to say on molecular orientation?

NEBEL: I believe that orientation of chromatic blocks is important in spiralization and may lead to random coiling. The ultimate molecular orientation within these blocks remains unchanged.

WRINCH: Are the molecular units stable; do they in fact maintain an invariable atomic pattern?

NEBEL: We know that in maize the visible blocks are stable and thus assume that others are also but we do not know.

WRINCH: What can be said about the sheath around the chromosome?

NEBEL: We need two sheaths, one for each coil; in the case of Swanson's observation, three.

METZ: The sheath is a layer directly around the chromonema; if the chromonema coils, the sheath coils too. I do not think of the sheath as a real membrane.

LASHIN: It appears that the chromonemata are capable of contracting into the form of a spiral with the aid of the surrounding sheath or membrane. In reviewing Chambers' work on *Amoeba dubia*, I noted that it seems that almost any type of stimulation will cause a contraction of the membrane as well as the protoplasm. This contracted animal may be at least half the size of the same animal under normal conditions. Since most membranes or sheaths display similar properties of elasticity, is it not possible that the contracted spiral chromonemata are largely the result of their contracted enveloping sheaths?

HUGHES-SCHRADER: In coccids, the outer layer is really sheath-like. At metaphase the chromosome sheath becomes continuous with the wall of the massive half spindle and with the wall of the stem body which appears later. As the sheath elongates the shape of the chromosome is changed. There is considerable range in this genus in the amount to which the sheath is differentiated. In one

species, the sheath is so rigid that on breaking the living cell each chromosome with its half spindles and stem body floats about stiffly in the medium for some time; in another, the sheath is delicate and not easily demonstrated. I have no evidence on the relation of the coiled chromonema to the sheath.

SPARROW: In a photograph not shown here, the matrix can be seen extending beyond the spiral and there is no doubt that they are optically differentiated.

GATES: The Feulgen and light green technique shows a green sheath around a red chromosome.

METZ: We are differing in what we call a sheath. In some cases, as in the chromosome vesicles in fish eggs or in some orthopteran cells, there appears to be a peripheral membrane which is said to take part in forming the nuclear membrane. I do not consider this to be the chromosome sheath.

GATES: In plant chromosomes, it is not related to the nuclear membrane.

HUSKINS: Until more is known about the matter, I am using the terms matrix and sheath without further definition to indicate respectively the material seen around the chromonema and its limiting membrane.

METZ: The term matrix, so far as I know, is used for the material between the gyres and in the axis of the condensed chromosome at mitotic stages, but is not applied to any material in the interkinetic stages when the chromonema is uncoiled. The term sheath has been applied to a layer of material around the condensed chromosome, and also to a layer of material around the extended chromonema in interphase. To me it seems most satisfactory to think of the sheath simply as a layer of gelated material surrounding and insulating the chromonema at nearly all stages. The material may be nucleoplasm or matrix (if this differs from nucleoplasm); its nature is secondary to its presumed insulating function.

PAINTER: What is the objection to the matrix as a core of material with a pellicle as membrane? Contraction would be initiated in the matrix.

HUSKINS: The matrix and sheath are absent in some heat-treated *Trillium* and the major spiral is then also absent.

SAX: Irradiated meiotic chromosomes often appear to have a ruptured pellicle which releases the major coil. This observation suggests that the normal pellicle maintains the chromonemata in the coiled state.

NEBEL: The pellicle must be demonstrated by physiological methods (see Dr. Buck's work).

SCHULTZ: The term matrix is not a good one, since the matrix is not a mold but something produced by the chromonema. Another term is desirable.

SCHRADER: Heitz uses *kalymma*.

METZ: I do not think the pellicle is a structure already formed and into which the chromonema is packed. But the pellicle is nevertheless a real structure.

BREHME: In Dr. Nebel's material, does he find the variability in appearance of the chromosome following different preparations, such as has been described by Dr. Metz for the salivary chromosomes of *Sciara*, and if so, how do you decide what is a normal image?

NEBEL: There is no normal image. Each treatment has its own standards.

HUSKINS: Because of this variability, chromosome measurements must be averages of large numbers and observations must be comparative.

DELBRÜCK: Huskins says he believes in chromomeres. What does he mean?

HUSKINS: Belling and others earlier (Schrader and Rhoades suggest Wenrich), saw chromomeres at leptotene and pachytene, which are argued by some to be optical illusions formed by a strand which is coiled and whose turns could give the impression of chromomeres. Nebel finds the same number of substandard somatic gyres as chromomeres in *Tradescantia*, but neither he nor I think the chromomeres are an illusion. The correlation has another interpretation.

METZ: Are the chromomeres lumps of material and not coils?

HUSKINS: Yes.

CARLSON: Isn't the question really whether chromomeres and spirals are present together?

HUSKINS: Some apparent chromomeres are due to turns in the coil but all are not.

MULTIPLE CHROMOSOME COMPLEXES IN ANIMALS AND POLYSOMATY IN PLANTS

C. A. BERGER

In these days when experimentation forms so large a part of the scientific method it is well to recall that observation is still the first step in all scientific investigation. This is especially true for the cytologist. New techniques and refinements of old techniques make it necessary to reinvestigate and to reinterpret the earlier findings of classical cytology. The first of the two cytological phenomena reported upon in this paper, multiple chromosome complexes in mosquitoes, was discovered, I am told, by Eleanor Carothers, and briefly described by a graduate student, C. M. Holt, in 1917, almost a quarter of a century ago. The improvements in cytological technique of the past twenty years, especially the aceto-carmin smear method and the Feulgen technique for small total mounts, have made the reinvestigation and the reinterpretation of this phenomenon a simple matter. Polysomaty in plants was discovered even earlier, by Stomps, in 1911. Since then a number of investigators have studied the process but it was not until 1940 when Gustafsson correctly interpreted polysomaty partly by improved techniques and partly by applying the findings of the multiple chromosome complex study in mosquitoes. Multiple chromosome complexes in animals and polysomaty in plants are of importance in this Symposium for two main reasons. In the first place they tell us something about the time of chromosome reproduction, secondly they serve as a valuable form of comparison for interpretations of giant salivary gland chromosomes. I will first briefly describe the multiple chromosome situation in mosquitoes.

MULTIPLE CHROMOSOME COMPLEXES IN CULEX

The ileum of a mosquito larva is a thin walled tube composed of a single layer of epithelial cells and an outer single celled layer of muscle cells. The phenomenon here described is found in the epithelial cells. Throughout the whole of larval life the ileum increases in size. This growth is brought about by a progressive increase in cell size, not by cell multiplication. The total number of epithelial cells in a fully grown larva is approximately the same as that of a very young larva. The increase in cell size is accompanied, in this tissue, by an increase in chromosome number from the diploid number six to such high multiples as 48, 96 and rarely 192 chromosomes. At the end of the growth period all the epithelial cells of the ileum are large but they are not all of the same size. There is a direct correlation between the size of the cell and the number of chromosomes it contains. Increase in cell size during larval life is a common occurrence in insects,

and in many cases it has been reported that these greatly enlarged cells are polyploid. The universal observation has been that these enlarged cells never divide but undergo cytolysis during the pupal metamorphosis of the insect. This is, in fact, what happens in many other tissues of the mosquito larva, in the colon and the mid-gut for example. The epithelial cells of the ileum are exceptional in this regard. Here the large cells do divide and the adult tissue is formed by the division and multiplication of these same larval cells. Throughout the whole of larval life the nuclei of these epithelial cells remain in what is apparently a typical resting-stage condition. No visible morphological changes in the nucleus, such as were described by Painter and Reindorp (1939) and Geitler (1938), give evidence of the endomitotic process which is taking place within them. After the long larval resting-stage of about ten days metamorphosis begins and these cells undergo a succession of divisions. At metaphase of the first of these divisions it is possible to determine directly the degree of polyploidy attained by counting the number of chromosomes. This procedure shows clearly that the largest cells have the highest degree of polyploidy and smaller cells have correspondingly lower multiples of the diploid number (Berger, 1938a). The prophase of these first divisions are especially interesting (Berger, 1938b). The chromosomes come out of the long resting-stage in the form of six bundles of closely associated chromonemata. Homologous bundles are loosely associated thus reflecting the somatic pairing established by Metz (1916) as characteristic of the Diptera. In favorable regions the number of sister chromonemata in each bundle may be counted. As prophase continues the groups of chromonemata contract and before metaphase is reached each group falls apart into separate chromosomes, each chromosome consisting of two chromatids united at an undivided spindle attachment region. The earliest prophase groups observed were about twelve times the length of the corresponding metaphase chromosomes. This contraction appears to be due to the formation of a major spiral in each chromatid arm since such spirals are plainly visible at late prophase and early metaphase.

The early prophase groups of sister chromonemata have a slight similarity to salivary gland chromosomes. This similarity is only superficial and the difference between the two structures are more significant than their resemblances. The prophase complexes of *Culex* are about twelve times the length of the metaphase chromosomes while salivary gland chromosomes are reported to be from seventy

to one hundred times the metaphase length. The *Culex* groups do not have the large amount of structurally differentiated achromatic material that is so characteristic of salivary gland chromosomes. Finally a multiple complex is unmistakably composed of a definite number of separate chromonemata while the structural composition of the salivary gland chromosomes is still uncertain, at least at this stage of the Symposium program.

POLYSOMATY IN PLANTS

The term *polysomaty* was introduced by Langlet (1927), to designate the presence of cells of varying degrees of polyploidy together with diploid cells in the same tissue. The phenomenon was discovered by Stomps in 1911, and studied by several investigators since that time. The more important recent papers are by Lorz (1937), Gentcheff and Gustafsson (1939), Ervin (1941), and Berger (1941). Polysomaty must be distinguished from several other types of polyploidy. Polyploid organisms are those in which the chromosome number is a multiple of the haploid number and all the cells of the organism have the same multiple number. Polysomy is the condition in which all the cells of the organism have the full diploid number plus one or more additional chromosomes. *Datura* is an outstanding example of polysomy. Cases of polyploid tissues are known where the organism is diploid but certain definite organs are regularly polyploid, for example, the tetraploid fat-bodies of certain insects. In polyploid tissues all the cells of that particular tissue or organ are of the same degree of polyploidy. Polysomaty differs from the above mentioned cases in that the organism is diploid, the polyploidy is restricted to certain tissues but the tissue also contains many diploid cells and the polyploid cells are of a variety of degrees of polyploidy, all however, multiples of the diploid not of the haploid number. The multiple complexes of the mosquito are more similar to polysomaty than to any of the other types.

Polysomaty has been most thoroughly studied in the root tips of *Spinacia oleracea* although it has been reported in five or six other plants from widely separated families. A brief description of the condition in *Spinacia* follows. All the common varieties of spinach are diploids with 12 chromosomes. One of the six haploid chromosomes is a SAT-chromosome. In the perilem of the root tip in addition to diploid cells many polyploid cells are present with $4n$, $8n$, and rarely $16n$ chromosomes. It is to be noted that this is the normal condition in all spinach root tips and is not the result of artificial treatment of any kind. Here too, as in the mosquito ileum, we are concerned with a normal developmental process. There is a definite correlation between the size of the cell and the degree of polyploidy. In the anterior region of the perilem, nearest to the tip diploid cells are found, posteriorly in

older regions of the growing point tetraploid cells and divisions occur. Still farther back from the tip octaploid and rarely 16-ploid cells are found together with smaller cells of lower degrees of polyploidy and diploids.

Polyploid metaphases are of two kinds: in one type the chromosomes are all in closely associated pairs, in the other type they are scattered at random on the metaphase plate with no evidence of pairing. The prophase of polyploid divisions are likewise of two types, some with paired and others with unpaired chromosomes. The late prophase stages of polyploid cells with paired chromosomes bear a striking resemblance to diakinesis figures and for this reason have been termed "somatic diakinesis" by Wulff (1936). Regarding their position in the root, paired metaphases are always found nearer to the tip than unpaired metaphases of the same degree of polyploidy. The chromosomes of a paired metaphase are slightly shorter than those of unpaired metaphases. It is to be noted that in spinach and in other plants manifesting polysomaty the chromosomes are never in groups of more than two, they are either single or in pairs, never in groups of four or eight as is the case in multiple complexes of the mosquito. This point is of special significance in the question of the origin of polysomaty.

Three classes of theoretical explanation have been proposed for the origin of polysomaty. In the first class are grouped explanations involving some kind of cell or nuclear fusion, pseudoamitosis or restitution nucleus formation. This type of explanation was popular with the earlier investigators, but has been abandoned by more recent authors for two main reasons. In the first place nuclear fusion does not account for the paired condition of the chromosomes found in many cells, and secondly the observational evidence for nuclear fusion is now correctly interpreted as a succession of stages in the transformation from telophase to resting-stage. The second class of explanations involve a double splitting of the chromosomes during prophase. This explanation must likewise be discarded since the demonstration by Gentcheff and Gustafsson (1939), that the chromosomes are already split at earliest prophase. Most probably the correct theory of the origin of polysomatic cells is that first published by Gentcheff and Gustafsson (1939) and held by the present author, that is, *double chromosome reproduction in the resting nucleus*. In favorable cases the chromosomes can be seen to be split at the earliest prophase of both paired and unpaired divisions, furthermore paired chromosomes are relationally coiled at early prophase which can only mean that they have come out of the resting stage in the paired condition.

Double chromosome reproduction in the resting nucleus also furnishes a satisfactory explanation of the paired and unpaired condition found in polyploid divisions. Metaphases and prophases with

paired chromosomes are those which follow a resting stage in which double chromosome reproduction has taken place. Unpaired metaphases indicate that one or more cell divisions have intervened since the doubling process occurred.

SUMMARY

By way of summary we may state that: 1) Both in an animal, the mosquito, and a plant, spinach, polyploid cells may arise by a process of successive chromosome reproduction within the resting nucleus. 2) Cell division and chromosome division (reproduction) are different processes which while they normally accompany one another are separable and hence distinct. The doubling of the volume of a cell does not necessarily lead to its division. 3) In plant material the double reproduction process occurs in steps with intervening mitoses; in animal material the doubling process may be repeated several times without intervening mitoses. This difference is clearly reflected by a corresponding difference in the prophase figures.

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DISCUSSION

DEMEREK: Is there evidence that multiple chromosome complexes in *Culex* are associated with division stages or do all divisions occur in the resting stage?

BERGER: No cell division occurs; the cells are very small and increase in size only.

GATES: Do nucleoli occur in these cells?

BERGER: I do not know the nucleolus situation in the mosquito.

PAINTER: In regions where the chromosome number may be counted, do you see any evidence of endomitosis?

BERGER: I have not seen it, but have not looked in other tissues than those described; it may occur.

DELBRÜCK: Does this process of chromosome complex formation lead to reduction in chromosome number?

BERGER: I believe it does. Somatic reduction seems to occur and to give rise to tetraploid tissue. I will not discuss this here.

METZ: Why not? This is very interesting from

the point of view of chromosome potentialities.

BERGER: One reason why somatic reduction may occur in this tissue is that the diploid number is so low.

SCHULTZ: What information is there about the centrosomes?

BERGER: None.

MULLER: Do you think the results of Jacobi on human liver are in agreement with your observations?

BERGER: I think so, but their work was done by measurement of cells which were not to undergo further division.

HUSKINS: Wermel later found that both growth and chromosome multiplication are involved in the liver cells.

CHILD: Where the chromosomes increase in number in *Culex*, do you get spindle fibers?

BERGER: There are no spindle fibers or any evidence at all for anything but a resting nucleus.

CHILD: When do you first find spindle fibers?

BERGER: At the beginning of metamorphosis when division of cells occurs.

STERN: Is it possible that the cells of the ileum are a mixed population with various degrees of polyploidy, from tetraploidy up? If this were granted, could one assume that the reproductive rate of the tetraploid cells is so much greater than that of the others as to result, after metamorphosis, in an ileum with only tetraploid cells? These assumptions would make unnecessary the postulate of somatic reduction.

BERGER: Cells of the ileum do not undergo histolysis—there is no evidence of histolysis; so they do not disappear, and the cells of the adult tissue must arise from them.

DELBRÜCK: Is somatic pairing carried through the earlier divisions?

BERGER: Yes.

DEMEREK: The condition in *Culex* and *Spinacia* is very different from that in the salivary chromosomes, where the compounds originate by division of chromonemata without separation and stay at prophase or metaphase, not resting stage, while in *Dr.* Berger's material division occurs in resting stage.

BRYSON: Darlington has described the salivary condition as an "arrested prophase" stage.

BERGER: The salivaries are not in metaphase.

DEMEREK: Their condition can be called prophase, but they would surely not be condensed if they were in the resting stage.

METZ: If they were in metaphase, they would be contracted, which they are not. So probably their condition is comparable to early prophase or resting stage.

SCHULTZ: There is no real contradiction, since the salivary gland chromosomes grow without separation of chromatids.

HUSKINS: The *Culex* case would be considered intermediate between normal division and the sali-

vary condition, a step with more than two chromatids associated.

SCHULTZ: Another intermediate step would be the nurse cell endomitosis described by Painter and Bauer in the Dipteran ovary.

BERGER: The difference between the *Culex* and salivary cases is that in the former separation of the chromatids occurs, in the latter it does not.

PLOUGH: The association of the salivary chromosome elements is much closer than anything Dr. Berger finds.

FANO: Is the polyploidy in Dr. Berger's material always a power of two (that is, 2^n) or is it ever a multiple of three (for example, 3×2)?

BERGER: It is not in multiples of three; reproduction is always double.

SCHULTZ: What is Dr. Berger's opinion on the polyploid condition in wounded plant tissue? May these not be results of a process like Levan's onion root tip endomitosis following auxin treatment? I wonder whether all these cases may be different forms of endomitosis?

BERGER: Probably this process is very widespread. (To Huskins): Is your figure from sorghum really an endomitotic condition or a wound phenomenon?

HUSKINS: In that particular case it was apparently a wound; but the whole tissue must be considered in determining the origin of such patches of polyploid cells.

POLLISTER: Do the nucleoli fuse to form a single nucleolus in octoploid cells?

BERGER: In spinach, there is always one large nucleolus. The nucleoli must fuse to form one while the cell is in the resting stage.

POLLISTER: In snails, the nucleoli fuse only at chromosome synapsis. Does this mean that Dr. Berger's chromosomes are really in synapsis?

BERGER: If this were so, diploid tissue would have to be in synapsis also, since there is one nucleolus there too.

GATES: Normally in root tips the nucleoli have generally fused by the time of prophase.

POLLISTER: In snails atypical spermatocytes occur where there is no synapsis and the nucleoli remain separate, as they are in gonads and the somatic cells.

BERGER: In the onion root tip, more than one nucleolus is present, but there is also more than one SAT-chromosome.

GLASS: Is the somatic pairing a residual association due to prior divisions or is it synaptic, i.e., *de novo* and due to the attraction of homologues from a distance?

BERGER: If the latter, you would not have metaphases with unpaired chromosomes, yet these are numerous.

METZ: In spinach, division accounts for the pairing, in *Culex*, it is synapsis.

WARMKE: How does Dr. Berger account for paired and unpaired tetraploid plates in spinach—on a basis of intervening divisions?

BERGER: Yes.

METZ: Dr. Schultz' question as to whether all large nuclei are polyploid must be answered in the negative. The *Drosophila* spermatocyte has a large nucleus but is not polyploid.

PAINTER: The toad egg shows lampbrush chromosomes which are paired but in addition we have a separate mechanism for the formation of nucleic acid. Very early in germinal vesicle formation large numbers of Feulgen positive granules are found in the periphery of the nucleus and separate, at this time, from the lampbrush chromosomes. These chromatic granules act as centers for the formation of hundreds of nucleoli, the material of which seems to be thrown off into the cytoplasm. Brachet has shown that these nucleoli contain ribonucleic acid. The lampbrush chromosomes seem to be normal chromosomes except for the tiny processes which radiate out from them; the last I think are made up of matrix material.

HUSKINS: Is the pairing in *Culex* due to attraction or to relational coiling?

BERGER: I think the chromosomes were formed together and come out of prophase together.

HUSKINS: This seems to fit in with the idea of residual coiling from a previous division.

PLOUGH: Does Dr. Berger explain all insect somatic pairing as due to division and not synapsis?

BERGER: Just this case.

PLOUGH: Why not other forms?

POLLISTER: In reduction from the polyploid number in *Culex* several successive synapses must occur, at least at the centromere region.

BERGER: Because the divisions are not synchronous, it is hard to tell which division other than the first is being followed in any given cell.

WARMKE: An active association of the threads during prophase must occur in the mosquito, because the threads appear to lie at random in the previous resting nucleus.

BERGER: This may occur in the very early prophase stages in which I have not been able to observe the chromosomes.

METZ: I was originally very skeptical of this interpretation of Dr. Berger's, but I certainly think now that the somatic reduction does occur.

STRUCTURE OF SALIVARY GLAND CHROMOSOMES

C. W. METZ

Like many other topics on this Symposium, the present one could only be discussed adequately by taking several hours instead of the allotted one. In consequence, it is necessary to confine the account to a few aspects and to run over some of these very rapidly. Furthermore, since there are numerous members here, especially among the physicists and chemists, who are not familiar with the material under discussion, it will be desirable to include a general account of the morphological and developmental characteristics of the salivary glands and their chromosomes.

To begin with, it should be stated that nobody knows at present how the salivary gland chromosomes are constructed. There are various conflicting views on the subject. Indeed, I know of no biological subject which has given rise in recent years to more differences of opinion and more conflicting interpretations than the present one. This seems surprising in view of the relatively enormous size of the chromosomes. One would expect that in these chromosomes, above all, the structure would be clear. But the conflicting interpretations clearly reflect the fact that in spite of the giant size of the chromosomes the details of their internal structure are obscure and difficult to interpret. Another important fact is also brought out by the differences of opinion just mentioned. This is that *a priori* considerations have played an unusually large part in the formulation of most of the interpretations thus far advanced. In order to understand the present status of the subject, therefore, it is necessary to review briefly these earlier interpretations.

Special attention has been paid to the structure of the salivary gland chromosomes largely for two reasons: First, because there seemed to be an opportunity here to throw light directly on the physical nature of the genes; and second, because there seemed to be an opportunity to throw light on the structure of "ordinary" chromosomes. It is only natural, therefore, that in studying the giant chromosomes there has been a strong tendency to identify microscopically visible components as individual genes, or something representing individual genes, and also to see in these chromosomes chromonemata like those present in "ordinary" chromosomes. At least six different interpretations have been presented which are based on the view that microscopically visible chromonemata are present here. Four of these interpretations have been abandoned; two are still current. In addition a seventh has been proposed (in place of one of those abandoned) which differs only in that bundles of chromonemata, rather than individual ones, are said to be visible. These facts are mentioned, not by

way of criticism, but because of their significance in illustrating the complexity of the problem. A different type of interpretation, proposed by Metz and Lawrence, will be considered later.

ORIGIN AND GROSS CHARACTERISTICS OF THE SALIVARY GLAND CHROMOSOMES

Before discussing the earlier views it is necessary to consider the general nature of the salivary gland chromosomes. Giant chromosomes, of this type, are known only in the Diptera (two winged flies). They are larval structures, which grow to enormous size during the development of the larva and then degenerate at the onset of pupation. They have apparently lost the power of mitotic division. Similar, but smaller chromosomes are found in some of the other gland tissues. Although ordinarily referred to in the singular, each salivary gland "chromosome" actually represents a pair of homologous chromosomes intimately united side by side in somatic synapsis. The duality is often obvious in ordinary material, and is conspicuous in species hybrids where synapsis is usually incomplete.

Figure 1 represents a photomicrograph of a pair of fully developed salivary glands from a larva of *Sciara coprophila*, one of the fungus flies. In this genus the gland is flat, not tubular, and consists of two rows of cells, with the duct between, making it especially satisfactory for the study of living chromosomes and of whole mounts like the one illustrated. In *Sciara* the gland is laid down in an early embryonic stage and consists originally of undifferentiated embryonic cells in full number; subsequent enlargement is due entirely to growth of the cells, without multiplication (E. Kirkwood, unpublished). Some idea of the enormous growth may be secured by comparing the large nuclei in the broad part of the gland with the tiny dot-like nuclei in the ducts near their point of union (fig. 1). The latter are normal nuclei, capable of mitosis, and similar to the initial embryonic nuclei. Nuclear measurements indicate that in *Sciara* the nuclei enlarge to nearly two thousand times their initial volume, and in some cases more.

It is generally agreed that the enlargement of the chromosomes is proportional to that of the nuclei. In *Drosophila melanogaster*, Bridges (1935) estimated that the fully enlarged salivary gland chromosomes are from 70 to 110 times as long as the condensed metaphase chromosomes in spermatogonial cells, which total about 7.5 μ for the four chromosomes. In the moderately stretched condition ordinarily used for study, the salivary gland chromosomes here total about 1180 μ , or approximately 150 times the metaphase length. In Chirono-

mus, Buck and Melland (unpublished) calculate the dimensions of a single salivary gland chromosome (pair) as 20 μ in diameter and 150 μ in length. In *Sciara coprophila*, the diameter of the salivary gland chromosomes is less than that in *Chironomus*, but considerably greater than that in *Drosophila melanogaster*. In all species, of course, the measurements vary widely and depend in part on the age of the larva and the size of the particular nucleus used.

Aside from their giant size, the most conspicuous characteristic of the salivary gland chromosomes in stained material is their distinctive patterns of alternating chromatic and achromatic transverse bands (fig. 2). These patterns are due to the presence of chromatic disks, separated by intervening narrow zones or "disks" of material which is mostly achromatic. The specificity of pattern is due mainly to the differences in thickness and other structural characteristics of the individual disks, and to the differences in spacing of the disks. Each chromosome, and each region in a chromosome, has its own characteristic pattern, as shown by Heitz and Bauer (1933), Painter (1933), Bridges (1935) and subsequent authors. Many of the chromatic disks are granular and appear in optical section as transverse rows of granules; other disks, or pairs of disks, appear as if made up of small chromatin-covered vesicles with achromatic centers. Especial attention has been given to such structures because of their possible relation to genes or to the chromomeres found in ordinary chromosomes.

REVIEW OF EARLIER INTERPRETATIONS

A little more than 30 years after the pioneer observations of Balbiani in 1880, the giant salivary gland nuclei were studied by Alverdes (1912) with a view toward analysing the structure of the chromatic "spireme," now known to represent chromosomes (Heitz and Bauer, 1933; Painter, 1933; Kostoff, 1930). Alverdes interpreted the disks of the salivary gland chromosomes as individual gyres of a heavy, coiled chromonema undergoing disintegration. It is clear from Alverdes' account that this interpretation (now abandoned) was based

largely on the fact that other observers, shortly before, had described coiled chromonemata in "ordinary" chromosomes. Alverdes' view persisted for the next twenty years, until after the true significance of the salivary gland chromosomes was discovered. Then Koltzoff (1934) and Bridges (1935) independently described what they believed to be chromonemata of an entirely different type here, delicate and more numerous, winding about the periphery of the chromosome (pair). This interpretation was likewise based largely on theoretical expectation, and has since been abandoned.

Subsequently, Koller (1935), Bauer (1936) and Painter and Griffen (1937) advanced other interpretations, based on the view that microscopically visible chromonemata, in still larger numbers, are present in the giant chromosome not only at the periphery, but through the interior as well. In connection with these latter interpretations, which will be considered more fully below, it seems significant that the first has been abandoned by its author (Koller, personal communication) and that the other two are incompatible with one another (see Metz and Lawrence, 1937). Likewise, it seems significant that the interpretation of Painter and Griffen has been modified (Painter, 1939) so as to regard the microscopically visible structures as bundles of chromonemata rather than individual chromonemata.

Still more recently Calvin, Kodani and Goldschmidt (1940), introducing a new method of approach, through chemical pretreatment, have proposed an additional type of interpretation involving microscopically visible chromonemata in large numbers. As I understand it, this interpretation postulates the existence of two types of chromonemata here—a basic set of four relatively heavy ones, and numerous longer and more delicate ones. Emphasis is placed on the basic set of four, and in a later paper Kodani (1941) apparently abandons the idea that there are also longer and more delicate ones.

In examining the interpretations just enumerated it is seen that all but three are based on the view that chromonemata like those found in "ordi-

Figure 1. Photomicrograph of a pair of salivary glands from a larva of *Sciara coprophila* Lint., omitting the slender terminal portion. From a Feulgen preparation made by Dr. C. A. Berger. Ordinary somatic nuclei of "normal" size are seen as tiny specks, indicated by the arrow, in the delicate ducts connecting the two glands. $\times 48$.

Figure 2. Photomicrograph of parts of two salivary gland chromosomes of *Sciara ocellaris* Comst., from an aceto-carmine preparation.

Figures 6-15. 6, drawing of portion of a section through an insect egg to show details of the pattern formed by the yolk-spheres and the intervening cytoplasm; designed to aid in interpreting the patterns seen in the salivary gland chromosomes. 7-13, photomicrographs of portions of salivary gland chromosomes; all from aceto-carmine preparations. 7, 9, 10 from *Sciara ocellaris* Comst.; 8, from *S. reynoldsi* Metz; 11-13, from *Chironomus* sp. Magnifications: 7, 8, 10, 12, 13, $\times 1700$; 9, $\times 1500$; 11, $\times 2500$. 10, two slightly different focal levels in one chromosome. Figure 12 shows the end of a chromosome, pointing toward the top of the figure. The edge of this chromosome is stretched to the right, giving the thread-like, granular lines extending out from this side. Figure 13 shows a portion of a similar chromosome stretched lengthwise. 14, 15, photomicrographs of cytoplasm from salivary gland cells of *Chironomus* sp. See text for explanation of all figures.

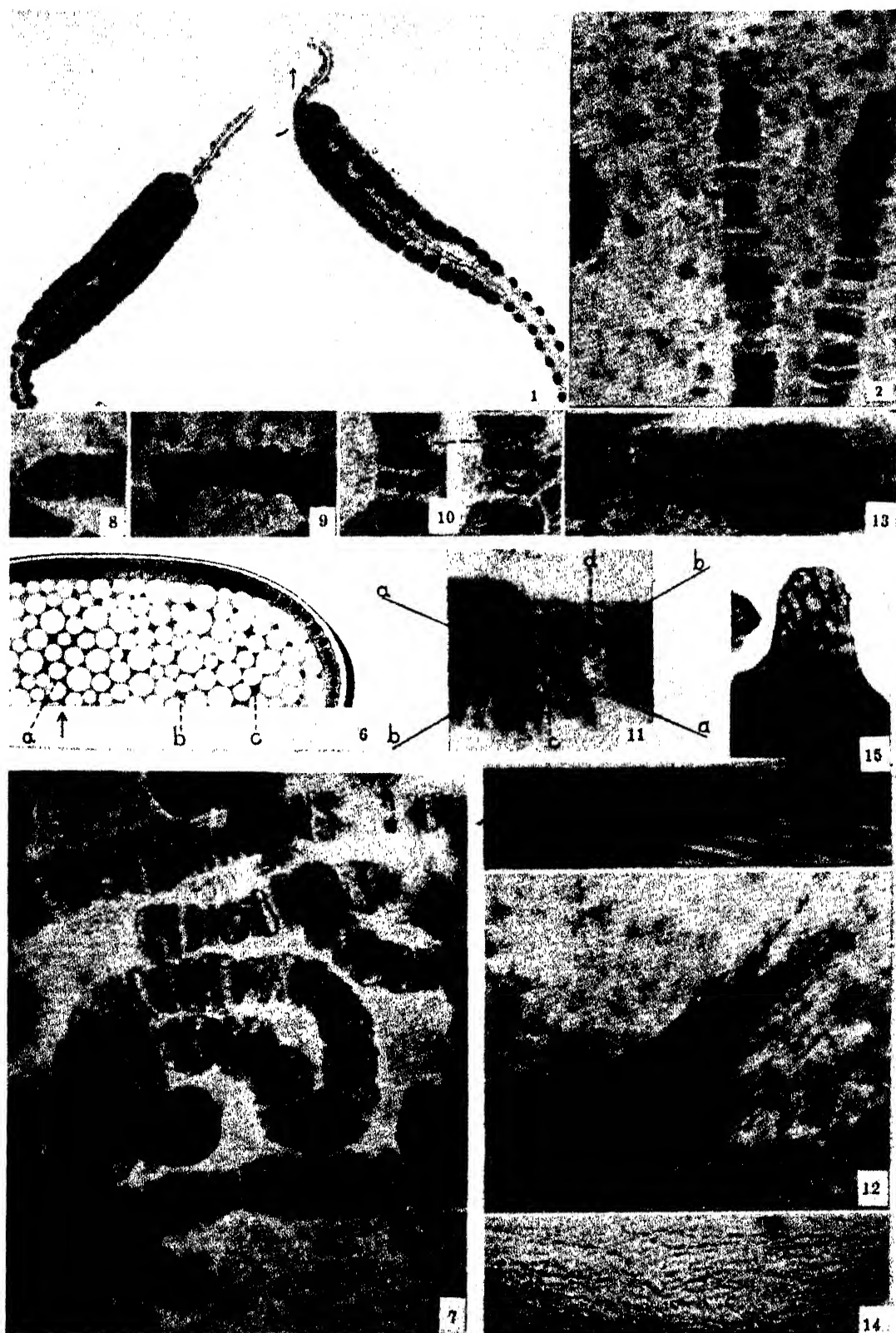


PLATE I (see facing text page for legends)

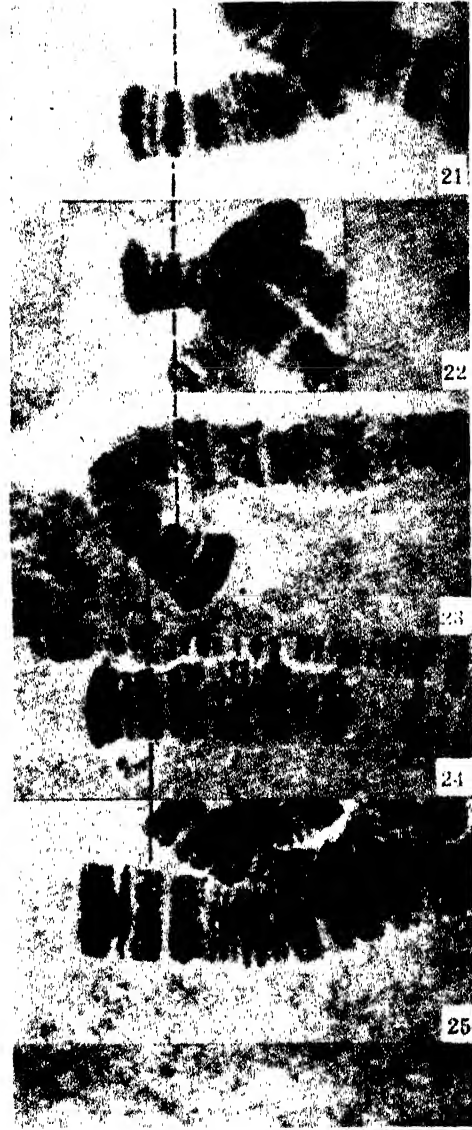
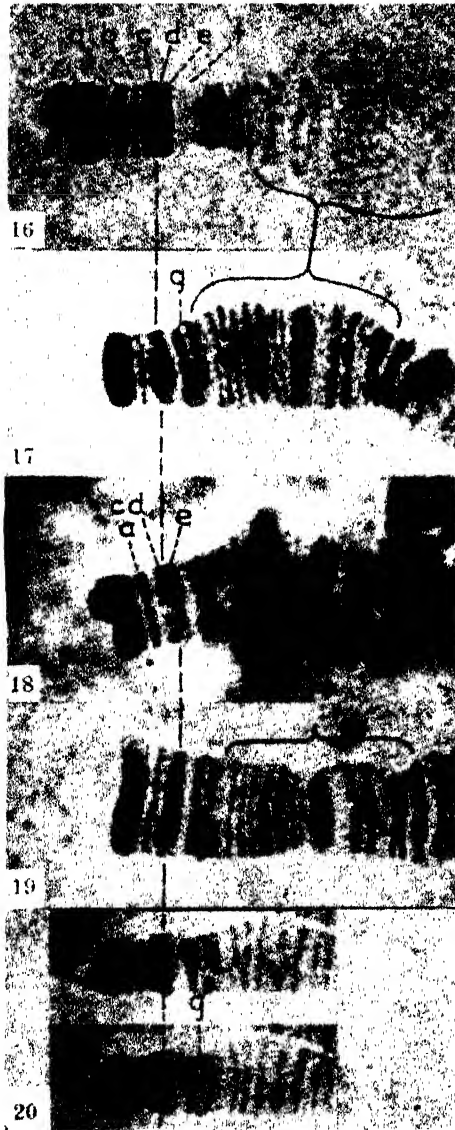


PLATE II (see facing text page for legends)

nary" chromosomes should be evident in the salivary gland chromosomes, and on the assumption that the latter chromosomes have arisen through a process of repeated multiplication of the chromonemata present in the initial chromosomes from which they developed. As noted elsewhere there are strong reasons for thinking that multiplication of chromonemata may form the basis of growth here. But would such chromonemata be microscopically visible in the salivary gland chromosomes? I think not. The underlying supposition is that during development of the salivary glands the chromonemata remain in their ordinary, normal condition, retaining their capacity for normal multiplication, even in the absence of cell division, and that the size of the giant chromosome or nucleus is an index of the number of divisions ("endomitoses") which have taken place during its development. It should be emphasized that this basic assumption does not permit hypertrophy of the chromonemata or the development of other abnormal characteristics. If hypertrophy is admitted the entire enlargement could be explained on that basis alone. If abnormal types of chromonema division or multiplication are postulated the basic assumption is likewise greatly weakened.

In the organisms under consideration the ordinary mitotic chromosomes are relatively very small (as compared, e.g., with those in certain plants) and it is difficult to estimate accurately the volume of a single chromonema. It seems safe to say, however, that a chromonema occupies no more than one fourth the volume of the metaphase chromosome, for there are at least two chromonemata present in such a chromosome, and probably half the chromosome consists of matrix substance. As emphasized in earlier papers (Metz and Lawrence, 1937; Metz, 1939), such a minute object, elongated to the length of a salivary gland chromosome, would be submicroscopic. On the basis of the measurements cited above, and others of the same kind, such a chromonema would occupy approximately one eight thousandth of the volume of a salivary gland chromosome. In volume it would be no greater than an individual granule in some of the disks. On the basis of such considerations alone, therefore, it is to be inferred that no chromonemata are microscopically visible in the salivary gland chromosomes unless we assume that they have become hypertrophied and are no longer "normal." If we make the latter assumption we may, as already noted, explain the entire enlargement of the salivary gland chromosomes on the basis of growth alone, without recourse to endomitosis. This latter

possibility is suggested by the recent observations of Kodani (1941). Painter (1939), on the other hand, holds to the conception of endomitosis, but attempts to avoid the consequent size difficulty by assuming that the chromonemata are present in bundles and that these bundles are microscopically visible. This is the basis for the modification of his earlier view (Painter and Griffen, 1937). Further consideration will be given to these views later.

The present account deals almost entirely with what is microscopically visible in the salivary gland chromosomes. Since it is impractical here to reproduce many of the photomicrographs with which the account was illustrated, as given at the Symposium, some of the features are merely described, or illustrated with diagrams. Drawings are not used, because it is felt that in material like this they reflect too much the observer's individual interpretation of what the structure should be. The numerous questions of theoretical interest must be deferred, in the main, for discussion in a later paper. Present interest centers primarily in the views enumerated above and the evidence on which they are based, together with certain structural features not dependent on any particular view. An attempt will be made to bring out all aspects in one connected account, rather than to discuss the different views separately. Since the effects of chemical pre-treatments, including the work of Calvin, Kodani and Goldschmidt are discussed by Dr. Painter elsewhere on this program, this aspect of the problem will be omitted here.

GROSS MORPHOLOGY

In *Sciara*, where the salivary gland chromosomes may be studied under high magnification in the uninjured living larva (Buck and Boche, 1938) the chromosomes ordinarily fill almost the entire volume of the nucleus under normal conditions (see also Doyle and Metz, 1935). This indicates that they contain within themselves most of the nucleoplasm. With slight changes in conditions which cause no permanent injury, such as reduction in the oxygen supply, a marked but completely reversible shrinkage occurs, involving loss of what appears to be essentially water and salts. This shows that the chromosomes may undergo extensive changes in volume under natural living conditions. In *Chironomus*, and probably numerous other forms, the chromosomes seldom occupy as much of the nuclear volume as in *Sciara*. When fully extended, in *Sciara*, the chromosomes show little internal structure. With shrinkage, the transverse bands become more and more conspicuous, and some of them appear

Figures 16-25. Photomicrographs of end 1 of chromosome B in *Sciara ocellaris* to illustrate structural characteristics and variations in details of pattern found in individual chromosome regions. The broken line connects the corresponding locus in all cases. Figure 20 includes two slightly different focal levels in one specimen. This is a single chromosome, not a pair like the others; hence its small diameter. All from aceto-carmin preparations. 17, 19 and 25 are from one pair of glands. With the possible exception of 22 and 23, all are $\times 1700$. See text for explanation.

distinctly granular. Ultraviolet photomicrographs reveal much the same characteristics in unfixed chromosomes in extirpated glands (Cole and Boche, unpublished). Upon fixation, extreme shrinkage is apt to occur, especially with the use of acetic acid, which appears to give this effect invariably. Thus most structural studies in this field have been based on chromosomes which have undergone considerable distortion due to shrinkage.

In addition to the achromatic material lost through shrinkage there is a large amount remaining in the chromosome after fixation. In the fixed chromosome, at least, this lies mainly in the achromatic zones or bands between the chromatic disks. There is much difference of opinion as to the physical state of this material in the fixed chromosome, as will appear below. The question is an important one because of the influence of the material on the structural pattern seen under the microscope.

Concerning the chromatic disks it should be made clear that, although interpreted as rings by some earlier observers, these are truly disk-like; that is, they extend entirely across the chromosome, transversely, like coins in a cylinder. It seems clear that the occasional appearance of rings or V's is due to secondary modifications of truly disk-shaped structures. What constitutes a single disk is not yet known. The terms "disk" and "band" are used interchangeably by most writers, and unless otherwise specified are used here in a purely descriptive sense to indicate something which looks single, but may be made up of two or more disks closely applied face to face. In many cases what appears to be a single disk in one nucleus may obviously consist of two or more in another. This does not mean that the basic number of disks is variable, but merely that closely apposed ones often cannot be individually distinguished under the microscope. How far this extends no one knows. It is possible that all the microscopically visible disks are compound, but I know of no good morphological grounds for making such an assumption.

Since attention will be given later to the wide variability in detailed structural pattern found in the salivary gland chromosomes, let me emphasize here my feeling that there is a constant, basic disk pattern, and that the variations observed are due to secondary modifications in the positional relationships of the chromatic and achromatic materials. It is true that some disks are so thin as to lie near the limits of microscopic resolution. This may mean that there are others too thin to detect, but it does not necessarily mean that the thicker ones are all compound. So far as I know, present evidence harmonizes with the assumption that some actually single disks are thin and others thick and that there is a considerable range between the two extremes.

INTERNAL ORGANIZATION

The pattern of chromatic disks in the salivary gland chromosome seems best interpreted on the as-

sumption that it reflects in some manner the basic gene pattern in the original chromosome, before enlargement took place, and that it is present on a miniature, submicroscopic scale in the ordinary counterparts of this chromosome in other cells. As already observed, the disk pattern is due to the size, form, sequence and spacing of the disks. The sequence and the size are features which do not concern us here, save as size may throw light on structure. The form and the spacing, however, are both important.

In a rough way four types of chromatic "disks" may be distinguished. In one the disk appears to be solid and continuous, forming a continuous, although not necessarily straight, band across the chromosome when viewed in optical section. In another the disk is granular, and appears in optical section as a row of granules, again not necessarily straight. As will be noted below, two adjacent disks may jointly contribute material to the formation of a row of granules. In a third type the "disk" is vesicular in makeup, being composed of achromatic droplets each covered with a thick coating of chromatic material. Opinions differ as to whether such a structure represents a single disk or a pair of disks (see below). The fourth type is that referred to by Bridges as "capsular." It appears to be fundamentally like the third kind just mentioned in that it contains droplets of achromatic material; but in this case the chromatic material instead of being distributed uniformly over the surface of each droplet lies mainly at the periphery of the composite structure so that the droplets are inconspicuous and the entire structure has the appearance of a capsule seen in longitudinal section (figs. 20, 23, at *g*). Here again it is often uncertain whether a single disk or two or more disks are involved. Needless to say, intermediate conditions are found between these four types.

A conspicuous characteristic of some chromatic bands is their tendency to follow clear cut zigzag paths, instead of straight paths across the chromosome (fig. 8). An individual band may be straight in one nucleus or preparation and zigzag in another. The significance of this feature appears when the nature of the material in the achromatic interband zones is examined. Most published illustrations of salivary gland chromosomes represent these chromosomes in what may be called the "sharply banded" condition. Here the bands are clear cut and relatively straight, and are separated by sharply delimited achromatic zones or "bands" almost entirely devoid of visible chromatic material (fig. 2). For genetic purposes of presenting the band pattern and mapping gene loci this condition is admirable, but its frequent use in publications has tended to obscure the fact that it is only one of a wide range of conditions in which the chromosomes are found. It appears, in fact, to represent one extreme in the series of conditions, each one of which is just as "normal" as the others.

At the opposite extreme, the chromosomes appear somewhat as shown in Figure 7. Here each chromosome looks as if it were filled with small achromatic droplets, bubbles or vacuoles, which distort the chromatic disks until many of them are scarcely recognizable. This has been referred to as the "vesiculated" condition (Metz and Lawrence, 1937 etc.). Aside from the vesiculated aspect, the most conspicuous characteristic of this condition is that here the chromatic material is not sharply limited to the bands, but much of it extends longitudinally from band to band across the achromatic zones, forming what appear in optical section as longitudinal lines. It is these lines which some observers have interpreted as chromonemata. As already implied, a complete range of intermediate conditions may be found between the strongly vesiculated and the sharply banded ones. Apparently the chromosomes in any nucleus may readily go from the former to the latter condition at least, and probably back and forth from one to the other, during their normal development. In *Sciara* the vesiculated condition predominates during much of the developmental period and the sharply banded condition tends to appear in later stages. The range of conditions may be secured from material all reared in the same manner and fixed and stained in the same manner. Whether the conditions observed in the fixed material are actually present in the living chromosomes before fixation is unknown, because it has not been possible to distinguish structures in sufficient detail for this purpose through the body wall of the living larva. In any event, however, it seems evident that the living chromosomes go through changes which are reflected by those found in the fixed preparations.

It is believed that the range of conditions just noted has particular significance in the present connection. The phenomena are interpreted in the manner indicated below (see Metz and Lawrence, 1937; Metz, 1939). Even in the banded chromosome there are certain "disks" which obviously contain achromatic droplets or vesicles (types 3 and 4, noted above). Whether these are single disks with droplets embedded in them, or represent two disks with droplets between, need not concern us at the moment. In many cases (type 3) the droplets are clearly surrounded by a layer of chromatin, giving the appearance of hollow beads. In other cases (e.g., type 4) there is less chromatin around the individual droplets, making them less conspicuous. In less sharply banded (i.e., more vesiculated) chromosomes, similar droplets appear in other regions, including the achromatic zones between different disks. In each case a single layer of droplets separates two adjacent disks or, in optical section, a row of droplets separates two adjacent bands (fig. 3A). Progressively this condition becomes evident in more and more of the chromosome until in strongly vesiculated chromosomes it is seen in nearly all parts. It seems clear that the achromatic droplets are

made visible by the presence of chromatin around them, apparently by dispersion or flowing out from the disks. We have been unable to find any real distinction between the picture exhibited by two disks with a layer of droplets between, and a so-called single disk with a layer of droplets embedded in it. The latter may equally well be interpreted as two closely applied, relatively heavy disks, partially separated by a layer of droplets, where the chromatin of the two disks extends over the surfaces of the droplets, giving them a bead-like appearance.

The behavior of liquid or jelly-like droplets when crowded together is well known, and is exemplified in many forms of protoplasm. An illustration from non-chromosomal material may help to make clear the conditions we are dealing with inside the salivary gland chromosomes. Figure 6 is from a drawing of an optical section through an insect egg, showing the yolk spheres or droplets and the continuous cytoplasm between them. Where a few droplets happen to be lined up in a row, the material between this row and the adjacent spheres on one side presents the form of a zigzag line with thickenings at the angles as indicated above the arrow. This line is, of course, simply an optical section of the continuous material between the spheres. Similarly, the short line extending from any angle of the zigzag, between two of the droplets of a row (e.g., that designated *a*) is an optical section of the continuous material. Where adjacent droplets are not so close together, as at *b* and *c*, the material between may be in the form of a concave-sided oblong or square instead of a line. All these configurations are found in the salivary gland chromosomes. Likewise, the six sided figures are found which are typical of tetrakaidecahedrons formed by droplets when pressed together (e.g. fig. 9).

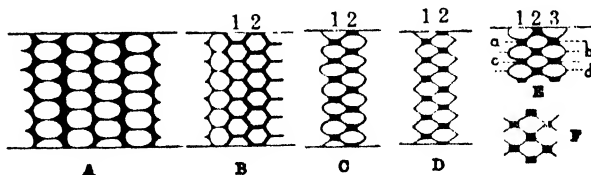


Figure 3. Diagrams illustrating various types of patterns seen in the salivary gland chromosomes, depending on the relative distribution of the chromatic and achromatic materials. In A the chromatin is located mainly in the disks (bands), which are separated from one another by achromatic droplets. In B the disks appear in optical section as zigzag bands, with the chromatin more evenly distributed over the surfaces of the droplets, giving a "honeycomb" type of pattern. In C and D the chromatin is aggregated into large granules or "chromomeres" between the disks, giving a checker-board type of pattern. E and F, small parts of patterns shown in C and D; see text for explanation. The numbers 1, 2, 3 indicate corresponding positions, between successive disks, in different diagrams. The diagrams in this figure are independent of one another and are not intended to represent a series, such as shown in Figure 4.

In the salivary gland chromosomes, however, the droplets are not scattered about at random, but are confined between the chromatic disks and appear in optical section as transverse rows, one row between each two adjacent disks. Except where separated by a relatively thick, heavy disk, the droplets of adjacent rows alternate with one another in position, giving a staggered effect. Correspondingly, the intervening chromatic band is wavy

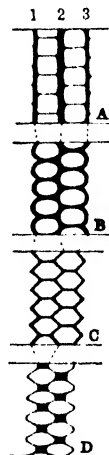


Figure 4. Diagrams to illustrate the transition from the sharply banded condition (A), through the condition in which the chromatin is more evenly distributed over the surfaces of the achromatic droplets (B and C), to that in which the chromatin is mainly in large granules between the droplets separating adjacent disks (D). In A, the chromatin is almost entirely in the disks, which appear as definite bands. In B, the chromatin is evenly distributed over the surfaces of the droplets, which retain their shape, and the disks appear as zigzag or scalloped bands. C is essentially the same, but the droplets appear six-sided in optical section, and the bands are all zigzag. In D, more chromatin has left the disks and collected in the spaces between the droplets, so the disks appear as thin zigzag lines, and the longitudinal lines connecting one disk with the next appear as oblong granules. The three chromatic disks (bands) are designated 1, 2, 3. The broken lines connect corresponding bands in the three diagrams.

or zigzag in outline (fig. 3B) like those just noted between the yolk droplets in the egg; and the longitudinal lines extending from one disk to the next, between the droplets of one row, alternate with those extending across adjacent rows, as indicated in Figures 3, 4 and 8-11. The distinctness of these longitudinal lines depends on the amount of chromatin surrounding the droplets. In the "sharply banded" condition, where most of the chromatin is in the disks, the lines are thin and faint; where much chromatin extends out over the surfaces of the droplets the lines are heavier; and in extreme cases so much chromatin extends between the droplets that the "lines" in some parts of the chromosomes appear as oblong or square granules with concave sides, like the spaces noted between the yolk drop-

lets of the egg (fig. 6, b and c). The series of conditions just cited is illustrated schematically in Figure 4. Individual regions in individual chromosomes are known to exhibit the complete range of conditions—from that in which the chromatin is mostly in the disks to that in which it is mostly in the intervening position in the form of oblong or square "granules" (see p. 29). Where two or more such transverse rows of granules are adjacent to one another they typically form a checker-board pattern as would be expected (figs. 3, 11). Further attention will be given to this in a later paragraph.

The inference drawn from such evidence as that cited above is that most of the achromatic material in the salivary gland chromosome, at least in the fixed condition, is in the form of droplets and that the size, form, number and position of these droplets is a major factor in determining the nature of the detailed pattern seen within the chromosome, particularly the granule pattern. Other factors, of course, are the thickness of individual disks (i.e., the amount of chromatin present), the pressure relations, and the distribution of the chromatin (i.e., the extent to which it is confined to the disks).

It should be emphasized that the distribution or dispersion of the chromatin, although greatly influencing the finer details of pattern, is very localized in extent. It is restricted to the zones immediately adjacent to individual disks and does not, so far as I can see, lessen the significance of the disks. Even where individual disks have lost their identity in the pattern, as in the regions showing checker-board patterns like that in Figures 3, 10, 11, 18, 19 and 21, the derivation of the block-like granules indicates that the granules are formed secondarily and that the fundamental organization is one of disks. What the distribution of chromatin between the disks does indicate, as brought out not only by the nature of the pattern, but by the kinds of variations found in it, is that the chromatin extends from one disk to the next in the form of what is essentially a continuum in which the achromatic droplets are embedded, and that the longitudinal lines between successive disks are optical sections of this material, like the lines between the yolk droplets cited above (fig. 6 at a), and are not individual threads or chromonemata. It may be that the "continuum" represents a mass of sub-microscopic chromonemata, packed together to make an optically continuous substance. This is what would be expected if, as seems probable, the chromosome is composed of thousands of chromonemata. Here again it should be emphasized that the conception outlined does not minimize the importance or significance of the disks. The fundamental constancy of the gross band pattern, the fact that ordinarily there is a single layer of droplets between each two successive disks, the relative uniformity in the thickness of different parts of any disk or band, the fact that the bands and the rows

of droplets separating them ordinarily extend directly across the chromosome, not diagonally or irregularly, and other lines of evidence, all combine to indicate that there is a fundamental disk organization. This concept of disk organization would correspond to Belling's "ultimate chromomere" concept (Belling, 1928) as developed from study of plant chromosomes, although the "chromomeres" he figured may not represent the ultimate ones any more than the microscopically visible disks here do, if as much.

✓ From the considerations just presented a conception of the salivary gland chromosome organization may be formulated somewhat as follows: Assuming, as is done by most observers, that the giant size is due to multiplication of chromonemata, we may picture the initial chromonemata in the usual manner as differentiated longitudinally into alternating short regions relatively rich and relatively poor in nucleic acid. Thickening, due to multiplication which would give a thousand or more such threads, would have the effect of transforming these regions into alternating disks. As multiplication of chromonemata proceeds and the nuclei enlarge, achromatic material, in the form of nucleoplasm, including presumably "sheath" and "matrix" material, accumulates. In the living condition a large proportion of this material is within the chromosome, and much remains even after fixation. Whatever its position in the living chromosome, this material appears in the fixed chromosome in the form of droplets lying mainly in the disk-shaped achromatic regions which contain relatively little nucleic acid—i.e., in the zones between the chromatic disks. It seems probable that this accumulation of achromatic material in these regions serves to elongate the chromosome, and accounts in part for the great length. Likewise, its localization in this manner serves to accentuate the chromatic disks, which would otherwise probably be so closely crowded together as to be indistinguishable from one another.

The picture thus formulated is, of course, subject to wide modification. As depicted, it implies that the "chromatin" in the chromatic disks is qualitatively different from that connecting one disk with the next, at least to the extent of containing a different amount of nucleic acid. This would presumably apply in the "sharply banded" condition, but less so in the "vesiculated" condition where part of the chromatin from the disks is included in the material connecting one disk with the next. Likewise, the picture requires the presence of two distinctly different components in the chromosome—the chromonemata themselves, and the more or less accessory achromatic material. In the absence of the latter, the chromonemata would form a solid bundle or cable in which the individual chromonemata would all be in intimate and uniform contact throughout their length (because of somatic synapsis). Since the individual threads

would be submicroscopic, each locus rich in nucleic acid would form a smooth, continuous chromatic disk, like a coin.

The presence of achromatic droplets or alveoli embedded in this structure, however, serves to alter the microscopically visible picture radically. Owing to the jelly-like, viscous nature of the material, the chromatic disks are modified in various ways to conform to the outlines of the droplets. They may be scalloped (figs. 4B, 5, 17 at *g*) or zigzag (figs. 3B, 4C, 8, 9) or may be broken up into granules, in which case the chromatin of two adjacent disks often combines in the formation of a transverse row of granules and the latter lie in an intermediate zone (see below).

Before going farther it is desirable to consider an actual case illustrating the range of variation which may be observed in an individual chromosome region. Figures 16-25 represent a series of photomicrographs of end 1 of chromosome B in *Sciara ocellaris* Comstock. The region of special interest is designated *c*, *d*, *e* in the first photograph (16); but before considering this, attention may be given to the terminal asymmetry shown in 16 and 18. The asymmetry is due to a small "deficiency," of the type common in this species (see Metz and Lawrence, 1938; Metz, 1938, 1941). One homologue of the pair lacks a short, apparently terminal, segment or region present in the other. It is interesting to note the difference in the appearance of this region in the two cases. In 16, it contains small droplets and is moderately stretched, showing longitudinal striations. In 18, it is relaxed and contains at least two large droplets or vesicles, giving a very different appearance.

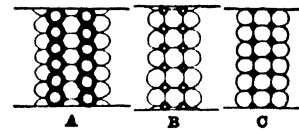


Figure 5. Diagrams showing different sizes of achromatic droplets with heavy coverings of chromatin, and the positional relationships between these and droplets with less chromatin surrounding them. When two rows of chromatin-covered droplets are separated by one row of droplets with less chromatin, as shown here, they give the appearance of chromomeres aligned on threads running longitudinally along the chromosome.

To the right of the heavy terminal region in this chromosome there is a series of bands or disks designated *a*, *b*, *c*, *d*, *e* (in 16). The first band (*a*) probably represents two or more disks; it is evident in all the figures, appearing usually as a row of granules. Next to this is a thin band designated *b*, which is seen distinctly only in the first figure (16). Then comes the region *c*, *d*, *e*, to which particular attention is directed. In 16, this region contains at least three distinct bands—*c*, *d*, *e*. Each of these may represent one disk or two or more;

that point is uncertain, for reasons already noted. The broken line connecting the different photographs designates this region (*c-d-e*) in the other cases. In 17, the details of this region are obscured by the dense stain; but heavy, block-like granules are present, as suggested near the upper margin. A similar condition is present in 18, and shows more clearly in the illustration. In the position *c-d* there is a single row of large, block-like granules and in the position *e* there is a row of similar, but smaller, granules, which alternate with those of the first row. The same condition is shown, even more clearly, in 21. If additional rows were present a checker-board pattern would be formed similar to that in the diagram (fig. 3 E, F). Figure 19 shows much the same condition in the upper part. In 20, two slightly different focal levels of one chromosome are represented. The structure here is similar to that in the last three cases cited, but the block-like granules are separated by large, chromatin-covered droplets (considered more in detail below). In 22, a somewhat different condition exists. Here the chromatin of the region in question is nearly all concentrated into one row of heavy granules,¹ instead of two. The other row is faint. In 23, 24 and 25, on the other hand, most of the chromatin of the region is concentrated into one solid, median band, with a thin band on either side of it—the latter seen best in 23 and 25. The achromatic droplets here are all small, as they are in 16.

Only two other regions in these illustrations need be mentioned. One is that designated *g*, which may appear in the form of heavy bands, as in 16, or a transverse row of conspicuous, chromatin covered droplets, as in 17 and 25, or a "capsule," as in 20, 22 and 23. The other is the long region indicated by the brackets in 16, 17 and 19. In most of the examples shown here this region appears normally banded; but in many preparations it is expanded into a "puff," as shown in 16, and to a lesser extent in 18. When puffed, the region appears finely granular, as in 16; all trace of bands is gone in some or most of it, and the edges often grade off into the cytoplasm, presenting a picture indistinguishable from that in the cytoplasm, as seen in 16. This subject has been treated more fully in an earlier paper (Poulson and Metz, 1938).

The variations in pattern just described may all be explained on the basis of changes, or differences, in the form, quantity and distribution of the achromatic material, without any essential change in the underlying chromonema organization. The tendency of the region *c-d-e* to produce or to include large achromatic droplets, in relatively small numbers, is a definite characteristic of this region. These large droplets are often covered with a relatively thick layer of chromatin and appear as vesicles or

"chromomeres" (fig. 20), sufficiently large so that by focussing up and down one may follow their contours. The droplets in the two rows alternate in position—i.e., are staggered—and the chromatin between them is commonly in the form of block-like granules in checker-board arrangement as shown in the illustrations (see also diagram, fig. 3C). In spite of the striking nature of this pattern, however, the evidence seems to indicate clearly that the pattern cannot represent a true picture of the basic organization in the chromosome. I see no way in which two rows of granules, in checker-board pattern like this, could be transformed into such patterns as those shown in 16, 23, 24 and 25 where three or more distinct disks are present. An original disk pattern of the latter type, however, could be transformed into the granular one, in the manner shown diagrammatically in Figure 4, by the accumulation of achromatic material in the form of large droplets between the disks.

The question as to just how many disks are actually present in the region *c-d-e* is immaterial at the moment. There are evidently at least three, and each of these may be at least double, but the two rows of large droplets when present appear ordinarily to lie in the zones *c-d* and *d-e* respectively. There may be much smaller droplets present elsewhere, obscured by chromatin. The condition represented in Figure 22, where most of the chromatin appears to be confined to one row of granules, may represent a somewhat different distribution of materials; but it may perhaps be better explained as due to a difference in staining capacity in band *e*. The second row of granules is present, but faint, somewhat as in 19.

The present interpretation of the checker-board pattern of granules, therefore, is that it is due to modification of disks, in the general manner indicated schematically in Figure 4, and described above. Owing to the viscous, plastic nature of the materials the chromatin of the disks (fig. 4A) may extend over the surfaces of the intervening droplets, to give an alveolar appearance (fig. 4B) or, with more crowding, a "honeycomb" appearance (fig. 4C) or may come to lie mainly in the intermediate zones, forming granules in checker-board pattern (fig. 4D). This aspect has been emphasized because, as will be seen below, the granules and the lines which connect them in diagonal rows in the checker-board, have been interpreted as parts of chromonemata by several observers. Likewise, the comparable diagonal lines in the honeycomb type of pattern (e.g., A-A and B-B in fig. 26) have been called chromonemata. Both the rows and the lines may be accentuated by stretching or distortion of the chromosome.

Additional examples, to illustrate the relation between the different kinds of patterns, are shown in Figures 11 and 10. The former is from *Chironomus*, the latter from *Sciara*. In the former (11) the alveolar or honeycomb pattern is shown in the

¹ Perhaps it should be noted that the photographs here have not been retouched in any way. The jet black, ink-like appearance of the granules in 22 is conspicuous in the photograph.

region *c* (see also fig. 9). It is difficult, of course, to find regions of this kind suitable for photographing, because the pattern is only distinct where numerous droplets all happen to lie in one plane and can be seen in median optical section at one focal level. This is seldom observed over more than a very small area in any one case, and is usually seen to best advantage at the bottom focal level where the chromosome is pressed against the slide and the image is not disturbed by anything at a lower level. In the region under consideration (at *c*) the chromatic bands are zigzag in outline, the chromatin extends over the surfaces of the droplets making (in optical section) short longitudinal lines of about the same thickness as the bands, the droplets appear in optical section as six-sided spaces, and those of adjacent rows alternate in position, so that a honeycomb type of pattern is formed like that shown diagrammatically in Figures 3B and 4C. It will be noted that the four disks in the region in question (at *c*) are about equal in thickness. The uniformity of the pattern is largely due to this fact. As soon as heavier disks are reached, as at the left and right, the outline of the droplets is no longer clearly revealed and the bands are essentially straight instead of zigzag. Extending diagonally across the honeycomb pattern (at *c*) and continuing in diagonal directions on both sides of this region, along paths parallel to those indicated by the lines *a-a* and *b-b*, are zigzag or granular lines like those seen in the diagrams (e.g., A to A, B to B, in fig. 26). These are the lines interpreted as chromonemata by some authors. In the honeycomb region they form parts of the honeycomb pattern, as just indicated, and are almost as distinct as the transverse bands, which contribute to their formation. Distortion of the chromosome often straightens such lines and makes them look thread-like. To the right of this region, in the zone designated *d*, the lines include granules of the checker-board type shown diagrammatically in Figure 3C and D. In this latter region the chromatin is mainly in the granules. The bands are thin zigzag lines, forming a crisscross pattern connecting the granules. The relation between this pattern and that of the honeycomb is indicated diagrammatically in Figures 3 and 4. The essential difference is that most of the chromatin in the checker-board or crisscross pattern lies in the spaces between the disks (in the gaps between the droplets) instead of in the disks themselves.

The relations between these different patterns is shown even more clearly, perhaps, in the photomicrographs of Figure 10. Here at two slightly different focal levels in a single chromosome region (indicated by the broken line) may be seen the alveolar or honeycomb structure at a low focus (shown in A), and the granules, connected by crisscross diagonal lines, at a slightly higher focus (shown in B). The relation between the components of the one pattern and those of the other, and the

derivation of the one from the other, are made evident by comparison with diagrams C and D in Figure 4. It should be emphasized that the picture showing the alveolar pattern (A) was taken at a low level, the bottom of the preparation, and the other at a higher level. Hence the former pattern cannot be interpreted as an optical artifact, due to granules at a lower level. Special care has been taken throughout these studies to guard against such misinterpretations. The granules in the second figure (B), of course, represent the short longitudinal lines, connecting the zigzag chromatic bands, in the first, and are called granules simply because they are thick. At this focal level more of the chromatin is in the position of the granules and less in the zigzag transverse bands, which on this account are almost indistinguishable as such; they are present, but the components of the zigzag make up the crisscross lines connecting the granules as indicated in the diagrams already referred to.

The diagonal lines making up the crisscross pattern in cases like those just described, have been interpreted as chromonemata, extending spirally in clockwise and counterclockwise directions. This is the conception, e.g., of Koltzoff and Bridges, and also of Bauer. On the first mentioned view (now abandoned) the clockwise lines would be considered to be at the upper surface of the chromosome and the counterclockwise ones at the lower surface, or *vice versa*. On the view of Bauer the two sets of lines would likewise be considered to be at different levels, but not necessarily at the surfaces of the chromosome. Actually, however, the lines visible at any one time do not lie at different focal levels and overlap; they are at the same level and intersect. This is shown by at least three lines of evidence: 1) Direct observation, by focusing the microscope up and down. 2) The fact that the lines intersect at the granules. If they were separate sets of granular threads the granules of one set would not coincide in position with those of the other set. 3) The derivation of the lines from those of the alveolar or honeycomb structure.

In other words, the lines in question, whether parts of a honeycomb pattern or of a granular, crisscross (checker-board type) pattern, are lines in a three dimensional, geometric organization viewed in optical section. The thread-like appearance is misleading. Three other lines of evidence, supplementing those just mentioned, also go to show that the lines do not represent threads. 1) In many cases the crisscross pattern is visible at all optical levels as one focuses up and down on the chromosome, in side view. Chromonemata, derived by repeated divisions of an initial set of four, could not give the picture seen. 2) If a chromosome showing the crisscross pattern is revolved 180° on its long axis, or intermediate amounts, the same crisscross pattern appears. If the former pattern were made up of threads, the threads would all be lying horizontally. Rotation through 180° would

put the threads into planes lying vertically and would give an entirely different pattern. And independent sets of crisscross threads extending in numerous planes such as would be required by the figures could not be derived by multiplication of the original chromonemata. 3) Individual lines in the crisscross extend across both homologues of the pair of chromosomes. The observations of Koltzoff and of Bridges are correct on this point, and the reason which was largely responsible for abandonment of their view weighs equally against its successor (that of Bauer)—namely, that a chromonema here would have to remain within one chromosome of the pair. It could not extend across both.

In this connection it may be said that through the kindness of Dr. Bridges and Dr. Bauer I have had opportunity to examine and discuss their material (*Drosophila* and *Chironomus*, respectively) in detail with them. Consequently, I am confident that the lines or rows of granules considered above are what they identified as chromonemata. I have also devoted much time to study of *Chironomus* material of several species, and to comparison of conditions in *Chironomus*, *Drosophila* and *Sciara*. These studies indicate that the chromosome organization is fundamentally the same in all three groups (also in *Simulium*, see below).

NATURE OF THE CHROMATIC GRANULES OR "CHROMOMERES"

As is well known, the chromonemata in ordinary chromosomes of many animals and plants often appear granular, somewhat like strings of beads. The granules are designated "chromomeres," ordinarily in a purely descriptive sense, but sometimes with the implication that the chromomere, if very small, represents a genetic unit, or gene (cf., Belling *l.c.*). Correspondingly, the term chromomere has been applied to the granules in the salivary gland chromosome, on the assumption that granular chromonemata are microscopically visible here. On this view the number of granules or "chromomeres" in any disk or at any one locus is considered to represent the number of chromonemata present, and hence to show how many chromonema divisions have occurred during the development of the chromosome from the initial stage in which four (two in each homologue) are present. On our interpretation, as outlined in the preceding section, this view cannot be correct. Nevertheless, since so much emphasis has been laid by other observers on the possible significance of the "chromomeres," the problem may be given further consideration at this point. An attempt will be made, 1) to recall certain difficulties inherent in the type of view just mentioned, in addition to those given in the preceding section, 2) to describe the different kinds of "chromomeres" observable, and 3) to indicate how the latter may be accounted for on the interpretation supported in the present paper. Items 2) and 3) will be considered together.

1) The view that microscopically visible chromonemata are present in the salivary gland chromosomes is subject to three obvious and serious objections, as already implied. One is that if the salivary gland chromosome is derived by multiplication of chromonemata there should be a thousand or more chromonemata present, whereas the number of granules in a disk is usually from five or six to 20 or 30 in *Sciara* or *Drosophila*, and has not been known to approximate the expected number in any material, so far as I am aware. Another objection is that the number of granules should be the same in the different disks of a chromosome (because the number of chromonemata should be the same in different parts of the chromosome), whereas the actual numbers are widely different. As indicated by the figures just given, some disks have several times as many granules as others in the same chromosome. Even if an organism were found in which these two requirements were met, the cogency of the objection would not be greatly lessened, for the requirements are general in application. To ignore them is to abandon the fundamental assumption on which the interpretation rests, namely, the assumption that the chromonemata have continued to undergo division here in the normal manner even in the absence of nuclear division. Ordinary reproduction of a chromonema is complete and rhythmic; such reproduction has occurred in each cell generation in the ancestors of these chromosomes over an extremely long period of time. To assume that in the salivary glands this process is suddenly altered so that, although reproduction continues in a definite manner, it occurs at widely different rates in different parts of a chromonema, is to assume potentialities in the chromonema for which we have no evidence elsewhere.

The third difficulty is seen in the size of the "chromomeres." If we assume that the granules etc., are components of individual chromonemata, we imply that each chromonema has undergone enormous enlargement, for some of the granules are greater in bulk than the entire original chromonema. Since this view requires the further assumption that these hypertrophied chromonemata undergo division, it involves two radical modifications of "normal" chromonema behavior. If we are to assume hypertrophy, it would seem preferable to account for the enlargement of the salivary gland chromosome entirely on this basis by simply assuming that, although growth continues, the capacity for chromonema division, like that for nuclear and cell division, is lost. Other difficulties will be brought out in later paragraphs. It should be noted that the objections just enumerated apply with equal force to the view of Painter and Griffen in its original form, and also, with the exception of item 1), to the modified form suggested by Painter (see below, p. 34).

2) and 3). In considering the nature of the "chromomeres" and the structural pattern observed

in the giant chromosomes the wide variations described above should be kept in mind. These variations help to account for the fact that different investigators have described such widely different kinds of "chromonemata" here. It is significant that even interpretations which have been abandoned have been presented by competent, careful investigators, on the basis of what is actually seen under the microscope as well as what was expected on *a priori* grounds. For this reason, any interpretation to be satisfactory now should be capable of explaining the phenomena previously observed.

The interpretation of Koltzoff and Bridges has already been considered. It was abandoned because it postulated chromonemata only at the periphery, and winding about both homologues of the pair. The view of Koller and Bauer (later abandoned by Koller) has likewise been considered. It is based on the same kind of evidence as that of Koltzoff and Bridges, namely, the presence of lines or rows of granules in relaxed chromosomes, transformed into more conspicuous lines in stretched chromosomes. But it recognizes the fact that such rows and lines are present in the interior as well as at the periphery of the chromosome. The nature of these lines and granules, and some of the reasons for not considering them to represent chromonemata, or chromomeres aligned on chromonemata, have been given in preceding paragraphs. Consideration of stretched chromosomes will be taken up later.

It will be evident from what has already been presented that granules of the type thus far considered are interpreted in the present paper as lumps, accumulations or concentrations of chromatic disk material in the spaces between the achromatic droplets, comparable to the block-like spaces (*B* and *C*) between the yolk droplets in Figure 6. As such, their occurrence would be, to a considerable extent, fortuitous. An individual granule may contain material from two or more chromatic disks, and the number of granules at any one locus varies with the number of achromatic droplets present, which is itself variable. Using the term chromomere in a purely descriptive sense, to designate a mass or lump of chromatin, these granules may be called chromomeres. Such chromomeres, of course, contain chromonematic material; but on the present view they would not be associated with individual chromonemata or individual bundles of sister chromonemata, and would have no significance as indicators of the number of chromonemata present or the genetic relationship of bundles of chromonemata (as postulated by Painter; see below).

Another type of "chromomere" appears to be distinctly different from that just considered. It is represented by the "heavy-walled" or "chromatin-covered" droplets mentioned above. Such a chromomere consists essentially of an achromatic droplet with a thick layer of chromatin over the surface. As already noted, certain loci, even in the "sharply

banded" condition, tend to show such chromomeres, and in "vesiculated" chromosomes other loci take on the same appearance. These chromomeres vary in size at different loci or at one locus in different nuclei, and range from a small fraction of a micron to one micron or more in diameter (in *Sciara*). Examples are represented schematically in Figure 5, and others have been noted in the photomicrographs (e.g., fig. 17, at *g*, and fig. 20 at the broken line). When very small these chromomeres often appear bead-like and solid as in figure 5C and possibly in band *a* in Figure 16. The solid appearance seems to be due merely to the small size of the droplets and the thickness of the chromatic coat. Achromatic droplets which do not have thick coatings of chromatin are ordinarily not referred to as chromomeres; yet so far as our observations go there is no real distinction between these and the others, and there is no sharp line of demarcation to distinguish one class from the other on morphological grounds.

When the size of the droplets is fairly uniform for some distance in a chromosome, as in diagrams A, B and C of Figure 3, in A of Figure 5, and at *c* in the photomicrograph in Figure 11, they tend to be staggered in position and to exhibit the alveolar or honeycomb pattern already described. Sometimes, however, a condition such as that shown diagrammatically in Figure 5C, is found. This gives the appearance of small, spherical granules aligned longitudinally—a condition emphasized by several observers. These granules seem clearly to represent the bead-like type of chromomeres just mentioned—i.e., small droplets of achromatic material covered with chromatin. Similar, but progressively larger chromomeres of this kind are also found, and are clearly droplets or vesicles of the type just mentioned. Examples are represented in diagrams A and B in Figure 5. In these various cases the arrangement of the droplets or chromomeres follows the same principle as that found in the honeycomb pattern. The droplets of adjacent transverse rows tend to be staggered in position, whether large or small, dense or pale.

Especial emphasis has been laid on these spherical vesicular chromomeres by Painter and Griffen (*l.c.*) and Painter (1939). According to these authors, if I understand them correctly, the vesicular chromomeres and likewise the granules or chromomeres of the type found in the checker-board or crisscross pattern described above, are aligned on chromonemata which tend to run longitudinally rather than diagonally (spirally) along the chromosome. The essential difference between this type of view and that of Koltzoff and Bridges or of Bauer, is indicated schematically in Figure 26. The stippled diagonal lines *A-A* and *B-B* in this figure represent what Bauer designates chromonemata; the stippled material between *C* and *C* represents a chromonema of Painter and Griffen, in which 1, 2, 3, etc., are considered to be chromomeres. In a chromosome re-

gion in which granules are prominent the two interpretations would be correspondingly different. For example, in Figure 3E the diagonal rows of granules would represent chromonemata on Bauer's view, while the combinations of alternating granules and droplets indicated by the dotted lines *a*, *b* etc., would represent chromonemata on the view of Painter and Griffen.

Recognizing the fact pointed out above, that if the salivary gland chromosome is made up of "normal" chromonemata these chromonemata would have to be submicroscopic and in very large numbers, Painter has modified the earlier interpretation (of Painter and Griffen). In its modified form the view agrees with that of Metz and Lawrence except in one important respect. It still regards what were called chromonemata on the former view as definite threads with chromomeres on them, but considers them to be continuous bundles of submicroscopic chromonemata instead of single chromonemata. If these "bundles" were considered to be irregular and essentially fortuitous associations, differing in a random manner in different parts of the chromosome, the interpretation would not differ significantly from that of Metz and Lawrence, supported in the present paper. But the interpretation calls for a special type of chromonema behavior by means of which bundles of specific composition are formed, and by means of which these bundles themselves undergo specific kinds of division, with genetic continuity throughout. Since such a view attributes to chromonemata potentialities which they have not been known to possess, so far as I am aware, it calls for careful scrutiny. Space will not permit a full discussion of the topic here, but a few points will be reviewed briefly.

As I understand it, the view is essentially as follows: Shortly after the initial stage in which four chromonemata are evident in the salivary gland chromosome (pair), eight "chromonemata" become visible; these give rise to 16, these to 32, etc. The ultimate number of microscopically visible "chromonemata" present at each locus is indicated by the number of chromomeres in the disk at that locus. These microscopically visible "chromonemata," however, are multiples, derived by division of preceding multiples in an accurate manner, so that the different bundles at one locus are alike. Since the number of chromomeres, and hence the number of bundles, at different loci differ widely, it is assumed that the bundles have correspondingly different rates of division at these different loci. The interpretation necessarily assumes a genetic continuity of bundles as well as individual chromonemata. And a sequence of 4, 8, 16 necessitates the assumption that each original bundle arose through multiplication of a single chromonema, either at the beginning of development or shortly thereafter. The chromomeres on these bundles are, of course, regarded as compound, each made up of a large number of identical components, each of which is, in turn, part of a single chromonema. The vesicular

chromomeres are regarded as chromatin-covered droplets like those described above. But the achromatic zone separating two adjacent bands made up of such vesicles is not supposed to include material in the form of droplets. It is simply supposed to be traversed by threadlike chromonemata, as indicated by line *E* and its counterparts in Figure 26.

It is evident that this interpretation of Painter does not agree with that supported in the present paper as regards the significance of the microscopically visible structural pattern in these chromosomes. Many of the objections to the views of Koltzoff, Bridges, Bauer, etc., have equal application here. For example, we have been unable to find any evidence of a sequence of 8, 16, 32 etc., chromomeres in individual disks during development. The numbers, which at best can ordinarily only be estimated, vary over a wide range, with no particular ones predominating, so far as we can determine. Although it is appropriate here to call the granules, vesicles etc. "chromomeres" in a descriptive sense, we find no evidence that they have genetic continuity and that each one divides as part of a continuous bundle of chromonemata, to give rise to two daughter chromomeres as parts of two daughter bundles, such as would be required on Painter's view. Among the other difficulties based on morphological evidence are those listed below. It should be noted that these do not depend for their validity on the acceptance of any other interpretation. They are equally serious whether the interpretation supported in the present paper is right or wrong.

1) The interpretation regards the chromatin granules described above, and also the chromatin-covered droplets or vesicles, both large and small, as chromomeres aligned on chromonemata (bundles). In some regions, however, both granules and vesicles are present side by side at one locus, as indicated schematically in Figure 3 E. At locus 1 in this figure one chromonema (e.g., that designated *a*) would possess a granule-chromomere while the chromonema next to it (*b*) would possess a quite different vesicle-chromomere. In other words, the constitution of the two homologous or sister chromonemata would be different at this locus and at all other loci where such a condition exists—which of course cannot be accepted.

2) Essentially the same difficulty is found in regions showing the "checker-board" type of pattern described above, as indicated in Figure 3D and F. The two diagonal lines extending out from each end of a block-like chromomere here have been interpreted as halves of a split chromonema, on the assumption that just beyond the granule the chromonema has undergone an additional division. On this basis there should be at the next locus two granules (chromomeres) for each one at the former locus. But in a condition like that illustrated, which is relatively common, the number of chromomeres is similar at adjacent loci, and forking lines extend from all the granules. To interpret these lines as chromonemata, if possible at all, requires the as-

sumption of a specific and complicated type of division, repeated from an early stage of development, such that the chromonema bundles at locus 1 (fig. 3F) all divide regularly along a different plane from those at locus 2, and no chromomere at the one locus involves the same bundle of chromonemata as any at the other locus. Each bundle at one locus would correspond to half of each of the neighboring ones at the next locus, as indicated by the stippled parts in 3F. The situation is further complicated by the fact that the condition described in this paragraph and that described in the preceding one may both be found at the same locus in different nuclei, e.g., the locus shown at *c-d-e* in Figures 18-21.

3) In regions showing an essentially uniform alveolar or honeycomb pattern, such as that shown at *c* in Figure 11, and in Figure 4B, the decision as to what should be called chromomeres is purely arbitrary. The interpretation calls for a series of chromomeres aligned on a thread, as indicated by the series 1-7 between *C* and *C* in Figure 26. As represented, a vesicular chromomere occurs at locus *D*

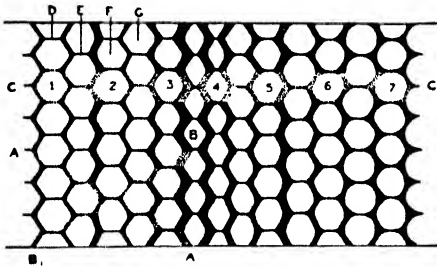


Figure 26. Diagram illustrating the alveolar or honeycomb type of pattern, and indicating the difference between the interpretation of Bauer (*A-A*, *B-B*) and that of Painter (*C-C*). See text for explanation.

and locus *F* (fig. 26), but in the similar regions or loci *E* and *G* there are supposed to be no vesicles or droplets. These regions are said merely to be traversed by threads connecting the chromomeres. The other chromonemata would have the same constitution. Obviously, however, the situation could just as well be reversed. The chromomeres could be at loci *E* and *G*, and the connecting threads at *D* and *F*. In other words, the enclosures *F* and *G* are alike and one could be a chromomere as well as the other; likewise there is no more reason for calling line *E* and the other lines at that locus threads than those at locus *D*.

4) A large part of the evidence used to support the interpretation is derived from severely stretched or otherwise distorted chromosomes. This will be considered briefly in the next section.

5) Although the difficulties already mentioned seem to be insurmountable, an equally serious one is seen in the nature of the supporting hypotheses required by the interpretation. If I understand the interpretation correctly it requires the assumption that in the salivary glands the chromonemata suddenly acquire the capacity for a complicated type of

collective behavior which ordinary chromonemata are not known to possess. To postulate repeated endomitoses, with continuous synaptic association of the resulting chromonemata, is in accord with what is known of dipteran chromosome behavior. But it is not in accord to assume that after a chromonema multiplies to form a bundle of 64 or 128 (approximately what would be required, e.g., in *Sciara*), the bundle then is capable of accurate division into two equivalent daughter bundles, followed, after another endomitosis, by division into two more equivalent bundles, etc. Likewise, it is difficult to see why, if the bundles divide, they should not undergo complete division rather than divide at different rates in different regions (as would be required because of the different number of chromomeres in different regions). The latter feature would require the assumption that in one region the bundles divide when they contain say 32 chromonemata each, at another region when they contain 64, at another 128, etc.

It would seem that such an interpretation as that just considered should be adopted only if clearly required by the evidence. In the present case, as already indicated, we feel that on the contrary the evidence supports another type of interpretation.

EFFECTS OF STRETCHING THE CHROMOSOMES

Unfortunately, this is one of the topics which can only be treated very briefly here. Nearly all the interpretations which postulate the existence of microscopically visible chromonemata in the giant chromosomes have been based to a considerable extent on evidence from stretched or otherwise distorted chromosomes. In the relaxed chromosome there is usually little to suggest the presence of continuous threads in any way resembling chromonemata. But in a stretched chromosome longitudinal or diagonal lines, distinctly thread-like in appearance, may be seen. The picture varies greatly according to whether the chromosome is in a "sharply banded" or a "vesiculated" or an intermediate condition. This is reflected somewhat in the different interpretations noted above. That of Bridges and that of Bauer emphasized the type of picture seen in "banded" chromosomes, where granules tend to be conspicuous and the checker-board or crisscross pattern is easily distorted into diagonal thread-like lines on which the granules are aligned (see Metz and Lawrence, 1937, fig. 22). That of Painter, on the other hand, emphasizes the condition in which droplets or vesicles are prominent, and in which stretching tends to produce longitudinal lines on which the heavy, chromatic droplets are aligned as "chromomeres." In all cases the chromatic material resists distortion more than the achromatic, so the paler, lighter parts of the pattern are the first to be distorted or disrupted.

Careful study of stretched and otherwise distorted chromosomes in *Sciara*, *Drosophila*, *Chironomus* and *Simulium* has convinced me that the lines called chromonemata are simply derived by distortion of

the patterns described above, together with disruption in various degrees when the stretching is severe. In many cases the derivation can be followed by studying a series showing progressively greater distortion. In *Chironomus*, Buck (in press) has shown by means of micromanipulation that the alveolar pattern may be transformed into the striated condition, in which the lines follow the direction of stress, and then return to its original appearance on release of the tension. The same principle applies in other cases; the lines follow the direction of stress longitudinally, diagonally or transversely within the limits imposed by the nature of the original pattern. These features may be illustrated by means of two examples.

Conspicuous thread-like lines may be made to extend directly out from the side of the chromosome by lateral stretching, as shown in Figure 12. Such lateral stretching is of interest also because of its failure to reveal longitudinal threads, which would be expected to be prominent under such conditions if any of microscopic dimensions existed. Figure 13 is from a *Chironomus* chromosome moderately stretched lengthwise. This chromosome, like that in Figure 12 (also *Chironomus*) is in a banded condition, with many granules; hence the lines are likewise granular. They extend in a longitudinal, slightly diagonal direction. In the left half of the Figure (13) the stretching is less severe than at the right, and the crisscross pattern of lines is evident, like those in Figures 10 and 11. To the right this is more and more broken up where the stretching is more severe.

A comparison with stretched cytoplasm aids in interpreting the effects of distortion on the chromosomes. Figures 14 and 15 are from photomicrographs of stretched cytoplasm in salivary gland cells of *Chironomus*. In 14 the cytoplasm is granular and forms thread-like lines on which the granules are aligned (compare with the chromosomes in figs. 12 and 13). In Figure 15 the cytoplasm is vacuolated and comparable with chromosomes in the vesiculated condition. The upper part of the figure shows an unstretched region; the lower part an adjacent region in the same cell where the material is stretched. At the lower right, greatly elongated individual vacuoles are indicated by the forking lines. This picture is much like that observed when vesiculated chromosomes, such as those shown in Figure 7, are stretched (compare with figs. 19 and 20 in Metz and Lawrence, 1937).

One of the most serious difficulties with the idea that the lines seen in stretched chromosomes are chromonemata, is the fact that their distinctness is in proportion to the severity of stretching, whereas just the reverse would be expected. If actual threads were involved, each thread in a severely stretched condition would be thin and relatively difficult to see. With less stretching it would be thicker and more conspicuous, and in an unstretched condition it would be most conspicuous—like a rubber band under comparable conditions. The actual

behavior is not like that of threads, but is like that of other forms of protoplasm in which, as the finer structure is distorted and disrupted, the viscous materials are drawn out into more and more thread-like lines as indicated in Figures 14 and 15. Ultimately, of course, stretching a chromosome produces strands of a really thread-like nature, and the chromosome material may even be stretched out into a single fine strand; but such strands cannot represent pre-existent structures or be chromonemata. According to our evidence, therefore, both the derivation of the lines in question from the geometric patterns discussed above, and the behavior of the lines themselves, indicate that the latter do not represent chromonemata or preformed bundles of sister chromonemata.

CONCLUSION

The view supported in the present paper is based on the assumption that the giant salivary gland chromosomes owe their size to multiplication of chromonemata which are present in very large numbers and are submicroscopic and therefore invisible. This assumption, however, is not based on direct evidence, but on theoretical considerations which cannot be reviewed here. A conceivable, although improbable, alternative would be the assumption that the initial four chromonemata have simply undergone enormous growth, without multiplication, to form the fully developed chromosome. The present interpretation would harmonize equally well with such an assumption.

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DISCUSSION

(Since discussion was invited during the presentation of this paper, some points included in the discussion were later covered in the paper.)

GREENSTEIN: Does the material in the bands stretch as much as that between the bands?

METZ: The interband regions stretch more, due to the fact that there is less chromatin in these regions.

PLOUGH: Is there any qualitative difference between the material in the bands and that between the bands, which would determine the amount of stretching?

METZ: I think the difference between the chromatin in the two regions is mainly quantitative—less chromatin allows more stretch. It seems clear that the true achromatic material, in the droplets, does not resist stretching as much as the chromatic material.

MULLER: May you not call the chromatic portion the continuous, the rest the discontinuous phase?

METZ: Yes, I think that this is a good way to describe it.

SCHULTZ: Another way of stating the question is that there are differences in concentration of staining material as between the bands and the interband spaces.

METZ: The disks of course are viscous, not rigid.

MULLER: Are there not different numbers of transverse rows of droplets in the same length of chromosome under different conditions, and would this not give a different number of disks?

METZ: I think there is no variation in the actual number of disks, even though the apparent number varies. I think the number of transverse rows of droplets is variable only to the extent that some rows do not always appear.

JOYCE: Are these chromosomes in the resting stage or do they show the changes in birefringence and viscosity which characterize mitosis?

METZ: They are not in a division process, but remain in one stage, probably the resting stage.

LURIA: Is evidence for concentration or precipitation of chromatic material (something resembling pycnosis) obtainable from following the chromosomes during metamorphosis?

METZ: There is no evidence for this.

SCHULTZ: Concerning the question of whether these same differences exist *in vivo*, it is known from ultraviolet photography that a picture similar to the fixed chromosome also is found in the living condition.

KAUFMANN: In heterochromatic regions in *Drosophila* salivaries, Bauer's description and my own material show presumed chromonemata traversing the nucleoli, especially when they are displaced by translocation; we also find ends of the chromonemata spread out. Can you explain these observations by alveoli?

METZ: I think there is no difficulty here. I have gone over Bauer's material, and Poulson and I made an extended study of the nucleoli in the same kind

of material (*Chironomus*). As we reported, thread-like lines may be seen in this material; but these extend out laterally, fan-like, with many branches, more than they do longitudinally. Our evidence indicates that they are not actually threads, and could not be chromonemata. Similarly, in the case of the spread-out ends of chromosomes, I do not think the lines represent chromonemata. The same kind of image is often seen at the edges of fixed cytoplasm, and much more thread-like structures are often found extending out laterally from the sides of chromosomes. These, of course, cannot be chromonemata. I think the lines at the spread-out ends are essentially like those seen within the chromosome and are to be interpreted in the same way.

BRYSON: Does the Feulgen reaction support the idea that chromatin lies between the alveoli in the achromatic regions?

METZ: Yes, stainable material is present there.

CHILD: From your slides it can be seen that all regions of the same chromosome in the same nucleus do not behave in a similar manner and also that the same region behaves differently in different nuclei.

METZ: Yes, variations of both kinds are found. Individual regions, however, have individual tendencies.

MULLER: What is a single band and what is a compound band? If this is hard to decide, it is also impossible to know the number of rows of alveoli. Are some alveoli too fine for observation by ordinary light?

METZ: I do not know any way of determining that a disk is single. When two disks are very close together the chromatin often extends over the intervening droplets or alveoli, obscuring them, and making what appears to be a single disk.

PLOUGH: How do you decide which alveoli are within a disk and which outside, i.e., how do you know when you have a disk at all?

METZ: The chromatic band goes straight across the chromosome and the disk is relatively uniform in thickness across the chromosome. The best interpretation is that the disks are real and uniform, otherwise the bands might go at various angles.

STERN: When a disk seems single at one time and double at another time, are the droplets between the elements of the visible double band present in multiple rows or is there a single row of large droplets?

METZ: Never more than one row in such a case.

WEBBER: How many chromomeres make up a single disk?

METZ: This varies widely.

WEBBER: If the disk is made up of multiple chromomeres, then a large number of threads must extend between the disks; since a small number of droplets are seen to lie between the disks, it might be interpreted to mean that the material between the droplets consist of chromonemata, since there is no other place for them to go. This concept does not disagree with Painter.

METZ: This is just the kind of interpretation I have been leading up to. If the number of chromonemata is so great, they would be equivalent to a continuous structure, or "continuous phase," but not discrete bundles having the power of accurate division, as on Painter's view.

CHILD: Are these stages reversible?

METZ: They appear to be reversible, because different pictures are seen at different stages of larval development.

STERN: Are these chromosomes really comparable to the living condition, or are these types of images due to treatment after removal from the larva?

METZ: All these images can be obtained with one kind of treatment.

WRINCH: The evidence relating to the existence of the disks in the salivary gland chromosomes seems to me to be of considerable interest in relation to structure, in that it demonstrates the high degree of specificity of the individual chromonemata. The alignment of the various regions, upon which the existence of disks depends, would seem to indicate a very definite atomic pattern on the surfaces of each chromonema. Such a situation is very reminiscent of many other parts of protein chemistry. It is like the close apposition of the faces of the insulin molecules in the crystals, of the faces of antigen and antibody molecules which form an antigen-antibody complex; it is also like the detailed fitting of atomic patterns which is exemplified by enzyme-substrate reactions. This is, in my opinion only one of the many ways in which the important findings of Dr. Metz and Dr. Painter bring the problem of chromosome structure into direct relation with the wider problem of protein structure in general. Another example is afforded by the plasticity of chromosomes in general which has been demonstrated. The comparison of protein crystals containing different amounts of water shows exactly this same phenomenon. Conjoined with a rigidity of atomic pattern in the individual protein molecules, required by the facts already mentioned, we are forced to postulate pliable interlinks to account for the relation between the various crystals. Particularly striking is the case of bushy stunt virus. In this latter case a very considerable difference in water content is shown in the so-called "dry" and "wet" crystals (both of which contain a large amount of water). I think this phenomenon, which can be studied in detail for this virus protein, throws light on the differing appearances of one and the same band in salivary gland chromosomes, upon which Dr. Metz has already commented. All these structures, virus proteins, chromosomes and even cytoplasm itself (as I shall suggest in my paper) are essentially protein-water systems, in all of which the same types of interlinks would seem to be present, and in all of which the essential structural entity appears to be characterized by a rigidly interconnected set of atoms on its surface, upon which specificity depends.

FANO: Concerning the interpretation of the micro-

photographs which have been shown in this and several preceding papers, I should like to emphasize the role of diffraction. The physical facts on this subject are these: whenever an image is formed of an object whose features are more detailed than one micron, there is bound to appear in the image an appreciable diffraction effect. The limitation to the correctness of images which is set by the resolving power of the instrument does not merely consist of the impossibility of distinguishing features which are too fine. On the contrary, diffraction effects can alter the image of objects which can be distinguished and they might even simulate the existence of features which have no reality. If, for instance, two threads are seen which are apparently well resolved and whose distance seems to be approximately 0.1 micron, we must not assume to have been able to push the resolving power beyond expectation, but we must think that those two threads probably are not real. For instance, if the width of a structure were two to four micra, and there were 10 alveoli within this width, the pattern of the alveoli might be determined by diffraction phenomena.

METZ: I am glad Dr. Fano has brought up the question of optical effects, for it is, as he says, important. I have, of course, observed such effects. So far as the structures considered here are concerned, however, I think there is no serious complication, for several reasons. For one thing, the chromosomes in the material I have used are much more than two to four micra in width. They range mostly from 6 to 15 micra in width. According to Buck and Melland (in press) the diameter of the living chromosome in *Chironomus* is 20 micra. Fixation would cause shrinkage, but flattening in making the smear would offset much of this. Another point is that the observations with visible light have been checked to some extent by using ultraviolet light. Although there is more to be done along this line, none of the results thus far gives any reason for questioning those obtained with visible light. Size considerations form one of the main reasons why I have preferred *Sciara* and *Chironomus* to such material as *Drosophila* for study of structural details. The granules, and especially the droplets, range up to a micron or more in *Sciara*. Some are as large as entire mitotic chromosomes of some organisms, and have a diameter not very different from that of an entire metaphase chromosome in somatic cells of *Sciara*. Although in some cases diffraction phenomena presumably do affect the sharpness of the alveolar pattern, I think it is clear that they are not responsible for the pattern. Furthermore, as already brought out, the interpretation of the patterns here is based not only on observation of individual images, but on a series of biological changes involving, for example, wide differences in the distribution of chromatin, which can be interpreted biologically and cannot, in my opinion, represent optical effects.

NEBEL: I have considered diffraction and refraction. Both must be taken into account; the latter is more important.

METZ: Some of the diagrams I have shown are superficially similar to the diagrams shown by Dr. Nebel, but actually, of course, the structure is entirely different. Some of the more delicate lines as in the stretched chromosomes are below resolving power, but the granules, droplets and other structures under consideration come well within the range of optical resolution. We have been well aware of the influence of optical patterns, and have made a special point of guarding against optical illusions.

PAINTER: To me the alveolar interpretation of salivary chromosome structure proposed by Dr. Metz does not make sense because it neither fits in with our ideas of the structure of mitotic and meiotic chromosomes nor does it jibe with the facts of salivary chromosome development. Let me make my ideas clear to you. I think that the bands of salivary chromosomes are made up of chromomeres; in side view in any optical plane these would appear as rows but in end view would remind one of a pepper-box, that is, the chromomeres extend clear through the chromosome. I think that each chromomere has a hull of chromatin surrounding a protein center. When the chromomeres are large the chromatin is squeezed to the free edges, and such a band, on Metz's interpretation, would consist of two chromatin disks separated by a disk of globules. Between the bands are the fibers which connect the chromomeres into longitudinal strands. These fibers are most conspicuous when the chromosome is stretched but in limited regions they show clearly in the lax chromosome.

In *Simulium* the chromomeres range in size from the barely visible up to 0.8 micron. Dr. Griffen and I have traced the development of salivary chromosomes from the initial four-strand stage. One must remember that before somatic synapsis each homologue is split. Each of these chromatids is chromomeric in make-up, that is, tiny chromatic granules are connected together by a thread. As the salivary chromosome (made up of four synapsed chromatids) increases in length and in diameter, more and more chromomeres become visible and the number of strands visible increases to 8, 16, etc. In short, you start development with four chromomeric strands and in the end you have a bundle of chromomeric strands.

Regarding the variability in the appearance of the bands which Dr. Metz has stressed—it should be remembered that the salivary chromosome is not static but is constantly undergoing change, as shown by differences within one gland. The differences may be interpreted as 1) due to the secretory activities of the cells; 2) due to the growth in size of the salivary gland chromosome; the only way proteins are known to grow is by reproduction of molecules each on the pattern of the others; chromosome growth must involve a reduplication process. In mitosis, there are changes in the water content of the chromosomes, and the nucleic acid content and nature of the matrix; the salivary gland chromo-

some probably undergoes these mitotic changes, presenting a prophase figure with corresponding variations etc.; probably changes in length and diameter are associated with endomitotic phenomena.

Nothing Dr. Metz says is not in keeping with a chromomere structure connected by protein fibers. The chromomere is a distinct entity from the earliest stage to the last stage.

METZ: Concerning the continuity of the chromomeres, you start with a chromosome composed of a few chromomeres and end with one with a large number of chromomeres along its length. I see no evidence of multiplication of chromomeres by division during development. The term chromomeres here seems useful only as a descriptive term.

PAINTER: The difference in number of chromomeres at different stages of development is due to the fact that the larger the chromosome gets, the more chromomeres can be seen. The pattern gets more complex as it enlarges. The chromomeres may originally be compound, and the chromonema coiled and later uncoiled.

KAUFMANN: The conflicting ideas of whether a chromomere is single or compound may be reconciled through another concept. X-radiation of *Drosophila* sperm produces chromosome breaks which are more frequent in certain regions than in others, and it has been suggested that the intercalary regions of high break frequency are heterochromatic. It may be that a few of the chromomeres we see are really heterochromatic regions spaced along the chromosome, while the true chromomeres are sub-visible in the early stages.

Concerning the gross reorganization of the salivary gland chromosome, four major strands composing each chromosome limb are sometimes seen in well-developed nuclei of larvae preparing to pupate. In such cases the halves of each of the homologues are often twisted as though relational coiling existed at that time.

HUSKINS: Much of the difficulty in reconciling the different ideas is due to the use of the terms of descriptive cytology for other aspects. Strictly speaking, there is no such thing as a "chromomere in a genetic sense"; chromomere is a descriptive cytological term. Again, visible splitting, not gene reduplication, is the cytological problem. The term gene is used in two senses, for the unit of heredity and the unit of development physiology, since for the latter we have at present no other name.

METZ: Much of this is what I have been trying to bring out. I do not believe that these "chromomeres" are bundles having genetic continuity, splitting to form four, eight, etc. I am glad to hear you emphasize the desirability of using the term "chromomere" in a descriptive, rather than genetic sense, even though some authors have used it in the latter sense, implying that they were dealing with self-reproducing structural units representing individual genes.

ENZYME STUDIES ON CHROMOSOMES

DANIEL MAZIA

The title of this paper is purposely ambiguous. In part, it deals with the action of enzymes on chromosome structure. The other part concerns the enzymatic activity of chromosomes. In no case, it is hoped, will chromosome chemistry automatically be equated with gene chemistry. If future discoveries justify such an equation, the situation will be a happy one. For the present, our crude notions of the chemistry of the chromosome may be seriously restrictive in our thinking about the genes. The problems of chromosome mechanics are important enough in themselves, and the chromosome, as will be shown later, may represent favorable material for the study of the problems of the gene reproduction.

CHEMICAL STRUCTURE OF CHROMOSOMES

Proteins. The facts concerning the protein analysis of the nucleus have been adequately reviewed (Gulick, 1938 et seq.; Mayer, 1938). The highly basic proteins, protamines and histones, have received a major part of the attention. It has often been stated that these are too simple to possess the versatility of gene material, but thymus nucleohistone, for instance, has been reported to have a molecular weight of over 2,000,000 (Carter and Hall, 1939). However, even the meager analytical data available point to the presence of other proteins in the nucleus. Huiskamp in 1905 obtained from thymus a "nucleoprotein X," which he believed to come from the cytoplasm. I have prepared this material and found, first, that it is strongly positive with the Feulgen reaction and, second, that the combination with thymonucleic acid is very stable. It seems likely that this is a protein of nuclear origin. Mayer has isolated from purified thymus nuclei a sulphur containing protein with an isoelectric point of about five.

This is all that chemical analysis has produced. The work does not even allocate the substances found to chromosomes, much less to parts of chromosomes. For finer study we turn to optical methods and histochemical methods. This paper represents the results of a combination of digestion methods with staining and ultraviolet microscopy.

The use of enzymes in histochemistry goes back to Schleiden (1839). In the study of chromosomes, nuclease was used by van Herwerden (1913) and trypsin by Caspersson (1936). With our present knowledge of enzyme specificity we can do much more than test for the presence or absence of particular kinds of substances. Since enzymes are now known to be specific for particular linkages (and even for particular linkages only when in specific relationship to other linkages) it is possible to apply enzymes to the study of *structures* rather

than substances. By observing the effect of specific enzymes on chromosome structure, we may evaluate the role of particular linkages in maintaining that structure. We have here potentially the finest of instruments for chemical microdissection.

Methods. The methods used are simple. Whole cells may be immersed in the enzyme solutions. Recently, I have found it possible to apply enzyme solutions to isolated single *Sciara* salivary chromosomes. After incubation, the chromosomes are observed with appropriate techniques. For nucleic acid studies, we used the Feulgen method and the ultraviolet absorption technique. Since there is no good color test for protein, although we have used the ninhydrin reaction, we have worked most extensively on material in which chromosomes may be most easily observed without staining. *Sciara*, *Drosophila* and *Chironomus* have been used for most kinds of experiments.

It has proved necessary to work at pH 5 or below even with enzymes with an alkaline pH optimum. In moderately alkaline solutions, the salivary chromosomes disperse into a fibrous mass. In strongly alkaline solutions, the dispersion takes the form described by Calvin, Kodani and Goldschmidt, (1940).

Effects of proteolytic enzymes. Trypsin. Trypsin completely digests and dissolves salivary chromosomes, as originally reported by Caspersson. The same effect has been observed with onion root-tip chromosomes and *Tradescantia* pollen tube chromosomes by my student, Miss Katharine Maneval.

Pepsin. Preliminary observations of the effect of pepsin on *Drosophila* salivary chromosomes were reported by Mazia and Jaeger (1939). The facts are 1) that the continuity of the chromosomes is not destroyed by the digestion and 2) that the chromosomes are drastically reduced in volume after digestion. The shrinkage takes place very rapidly in a 0.1 percent solution of commercial pepsin in 0.2 percent HCl. There is no effect apparent on the Feulgen staining of the chromosomes, except that it becomes more compact. Ultraviolet photographs have not yet been taken. The effect may be observed particularly well in isolated chromosomes held stretched in an agar gel. Here one observes a great lateral shrinkage, the longitudinal contraction being prevented. The shrinkage is greatest in achromatic bands.

I have in the past interpreted this experiment as indicating the presence in the chromosome of a matrix, composed of a different protein from the structural protein of the chromosome itself. This matrix protein is digestible by pepsin whereas the "skeletal" protein is not. The matrix protein occupies a considerable part of the volume of the

chromosome, and may account in part for the large size of salivary chromosomes. This interpretation is now confirmed by the ultraviolet absorption measurements of Caspersson (1940) who finds in the achromatic bands a concentration of a "protein of a globulin type," characterized by absorption around wave length 2800 Å. The pepsin indigestible "structural" protein would then be assumed to be a histone-like protein. The further evidence for this will be given below.

If the salivary chromosome is characterized by a high concentration of the "matrix" protein, it might be expected that the action of pepsin on active chromosomes might be smaller or absent. Miss Maneval has observed the effect of pepsin on onion root-tip chromosomes. A considerable shrinkage of the chromosome mass is evident, though details are difficult to make out in these preparations. Evidently even here there is some matrix though less than in the case of the salivaries.

Intracellular proteases. Papain. At the Gene Conference of 1940, the question was raised: why are not chromosomes digested when exposed to the enzymes of the cytoplasm? This question, of course, applies to any cell structure. The answers have so far not been satisfactory. In view of our growing knowledge of intracellular proteases, especially due to the work of the Bergmann laboratory, it is possible to attack this question.

In experiments with papain commercial papain was used, with both cysteine and HCN as activators. The conditions were those defined by Bergmann and Fraenkel-Conrat (1937). *Sciara* chromosomes only were used. No visible digestion was obtained either with activated or inactivated solutions at pH 5 for periods as long as 36 hours.

Tissue enzymes. Various tissues have been extracted and their proteolytic activity against chromosomes tested. The conditions were those defined by Bergmann and Fraenkel-Conrat (1937). Cysteine was used as activator. The following tissues were used: 1) beef liver, 2) pig liver, 3) beef spleen, 4) frog liver, 5) frog kidney, 6) frog testis, 7) autolyzing yeast. The results were almost uniformly negative. In only one tissue was an enzyme found which affected the protein of the *Sciara* chromosomes, and that was frog liver. The extract had to be activated by cysteine. The fact that this activation is required indicates that we are dealing with a typical intracellular protease, but why only the enzyme of frog liver was effective will be explained only when its activity against artificial substrates will have been studied. This information might give an important clue concerning the nature of the linkages responsible for the chromosome structure.

Tumor enzyme. Since dividing cells should be actively carrying on synthesis and breakdown of chromosome proteins (Caspersson, 1940), it seems likely that one would find in such cells a protease which affects chromosomes. Fruton, Irving, and Borgmann (1940) have found active proteases in

tumor tissues. I tried a sample of Jensen rat sarcoma, which was extracted with phosphate buffer at pH 4, activated with cysteine, and applied to *Sciara* salivary chromosomes. It produced no striking effects on the chromosomes; the tumor enzyme behaved as did the majority of intracellular enzymes.

Autolysis. Finally, an attempt was made to determine whether the salivary gland cells themselves possessed a protease to which the chromosomes were susceptible. Glands were placed under toluol for seven days, then stained by the Feulgen method. No digestion of chromosomes was observed to have taken place. It will be interesting to see whether glands of young larvae may not behave differently. Also, we should expect to find an effective protease in the pupa.

Protaminase. Weil (1935) has described in some detail a pancreatic enzyme which will split certain linkages in protamines, but which cannot digest true proteins. Since the presence of protamines in the nucleus is suspected, this enzyme has been applied to chromosomes. Actually, I used a crude preparation in which tryptic activity was inhibited by albumin and phosphatase activity by means of phosphate. After incubation, it was observed that the chromosomes were still present, but would not take a Feulgen stain. The effect is not due to phosphatase, nor to ribonuclease, since the latter is heat stable, while the active enzyme in my preparations is not. It seems probable that the effect is due to protaminase or to some unknown enzyme. Tests with protamine substrate indicated considerable protaminase activity in the preparation.

If we are dealing with protaminase in this experiment, it would seem that protamine-like polypeptides are present in the chromosome and that the nucleic acid component of the chromosome is attached to the protein through these polypeptides. Not much can be made of this experiment until we use a pure enzyme of known specificity. It is interesting, however, to find that an enzyme which apparently is not a nuclease can remove nucleic acid from the chromosome without affecting the fundamental structure of the chromosome.

Interpretation and summary. In interpreting these experiments, we must first inquire into the meaning of "digestion." In a solution, digestion has taken place when one chemical linkage has been split. The "digestion" of a *structure* means something else. It means that enough of the architecturally important linkages have been split so the structure is either radically altered or dissolved. If we are interested in the molecular architecture of the system, our digestion method becomes a method for evaluating the responsibility of particular bonds, recognized by their susceptibility to enzymes of known specificity, in the maintenance of the structure. For instance, it may be true, though it probably is not, that papain splits as many peptide linkages in the chromosome as trypsin. But the

bonds for which trypsin is specific may be those which hold the structure together. Such a possibility can be tested on a model system.

Since the simplest structural model for a chromosome is a nucleoprotein *fiber*, I have undertaken to compare the digestion of chromosomes with the digestion of a variety of artificial protein fibers. A very simple technique, based on the findings of Derivichian (1939) and Langmuir was used to prepare the fibers. Derivichian had found that a good surface film of almost any protein could be made by dropping on the surface an aqueous solution containing added surface-active material, such as amyl alcohol. Langmuir has shown that a completely compressed protein film becomes an elastic fiber or bundle of fibers, which is very easy to handle.

All attempts to prepare a fiber of protamine failed, as predicted by Wrinch (1936). Thymus histone and thymus nucleohistone formed excellent fibers. Thymus "nucleoprotein X" formed good fibers. These were compared with fibers of albumin and casein. All of the fibers were digestible by trypsin. All but histone and nucleohistone were digestible by pepsin. The behavior of histone mixed with other proteins, especially "nucleoprotein X" was studied. The molecules were mixed in solution. When pepsin was applied to the mixture, part of the material of the fibers went into solution immediately, and the rest contracted. In a few cases the contraction threw the residual fibers into spiral form. The contraction following digestion of one constituent is, of course, characteristic of the behavior of the chromosome. But the perplexing problem is the origin of the continuity of the indigestible portion. One possible interpretation, applicable to the chromosome, is that in the mixture we actually have a chemical combination of the basic histone and the more acidic protein. The film formed is, then, a film of the histone "salt," and the fiber is a histone fiber much inflated by the other protein. When the latter is digested, the fiber contracts.

All of the other proteolytic enzymes used on chromosomes have been applied to these fibers. It was found, astoundingly, that no enzyme tried, other than pepsin and trypsin, has any visible effect on any of the artificial protein fibers. It would seem, on the one hand, that fibrous organization alone would explain the resistance to digestion of the important structures of the cell. It would seem, on the other hand, that the enzymes of the digestive system represent a remarkable biochemical adaptation to the needs of the organism.

If information on enzyme specificity may be extrapolated from artificial substrates to fibers and chromosomes, the data may be interpreted. Since trypsin digests chromosomes and all the fibers, and since this splits peptide linkages neighboring free basic groups, such linkages must be vital in holding all protein fibers together. In the case of a basic protein like histone, trypsin should be most effective,

as it is. Pepsin, on the other hand, is active against linkages between certain amino acids in the neighborhood of free acidic groups. It would not be expected to be effective against histone, and it is not. This is more direct evidence for the histone nature of the continuous structural protein of the chromosome.

The inactivity of the intracellular proteases raises interesting questions. Certainly these split many bonds in a solution of a protein like albumin. Is there some physical condition which prevents their attack on the same bonds in a fiber? Or are these bonds so placed in the fiber that the fiber continues as such even though many peptide linkages are opened? Are these conditions which do not seem to favor digestion favorable to synthesis? This is the most interesting question of all. If the enzyme simply cannot attack the fiber, then, so far as the enzyme is concerned, the concentration of protein is always zero, and this, of course, would tend to shift its action in favor of protein synthesis.

However, as Bergmann (1939) points out, not only the physical conditions, but also a slight difference in chemical configuration may determine that an intracellular protease shall act synthetically. It might be that the linkages in the protein fiber possess such characteristics, but in examining the published data I have not found any substrates which are synthesized by intracellular proteases and digested by trypsin or pepsin.

The evidence for a continuous histone-like fibrous structure in the chromosome seems fairly complete, except for the unexplained case of the frog liver intracellular enzyme which digests salivary chromosomes but not fibers. The existence of the matrix of more acidic protein seems also probable. The picture of the interrelations of these fits very well the picture of Caspersson (1940). The evidence for protamine-like molecules associated with the nuclei acid is less convincing.

Chromosome proteins in plants. In the experiments on the digestion of plant chromosomes now being carried on by Miss Maneval, the results obtained generally parallel our data on salivary chromosomes. It has, however, often been stated that "nuclear" proteins, protamines and histones, have not been isolated from plants. The nucleoproteins of plants have, in fact, been neglected. Kiesel and Belozerski (1934) found evidence for the presence of both ribonucleoprotein and desoxyribonucleoprotein in bean seedlings, but do not classify the protein. Belozerski (1939) has found that the onion bulb contains no ribonucleic acid, but only desoxyribonucleic acid. If this is the case, this is suitable material in which to seek nuclear nucleoproteins. I have begun such an investigation, and, following the techniques (Kossel, 1928) used on animal material have obtained a small amount of nucleoprotein. The material has not been analyzed quantitatively, but its qualitative reactions are as follows:

it gives a strong Feulgen reaction, a positive ninhydrin reaction, a positive Sakaguchi reaction for arginine, a weak Millon reaction.

Fibers have been prepared and subjected to digestion. Their behavior is histone-like; they are digested by trypsin but unaffected by pepsin. The ultraviolet absorption spectrum of a solution in 0.01 N NaOH has been determined. The expected peak at 2650 Å is absent, indicating a relatively low concentration of nucleic acid in the protein. The nucleic acid content may well have been reduced in the process of purification by repeated precipitation in acid and resolution in alkali. The protein absorption is more pronounced, and is shifted toward wave lengths longer than 2800 Å. This shift is found by Caspersson to be characteristic of proteins of the histone type. In fact, the curve for the onion protein resembles closely some of Caspersson's curves for nucleohistone preparations. There seems, therefore, no good reason for assuming any great difference between the chromosome proteins of animals and plants.

Nucleic acid in chromosome structure. Since thymonucleic acid may form large elongated molecules (Signer, Caspersson, and Hammarsten, 1938), nucleic acid has been assigned various roles in maintaining the continuity and the apparent fibrous structure of chromosomes (Wrinch, 1936; Gulick, 1938; Astbury and Bell, 1938). If chromosome structure were in any way dependent on the nucleic acid component, the structure should be destroyed when the nucleic acid is removed. Miss Jaeger and I succeeded in removing it by application of beef spleen "nuclease" (van Herwerden, 1913). In *Drosophila*, we found, after digestion, that the carmine staining material had been removed, but the protein part of the chromosome, demonstrable by the ninhydrin reaction, was still present in its characteristic form.

It has since been found that *Sciara* is much more satisfactory material, since it is easy to observe the chromosomes even after they will no longer stain. Some evidence of banding is preserved, indicating the expected difference in protein distribution in the different bands. Very recently, Mr. Roman and I have taken ultraviolet photographs of isolated chromosomes after nuclease digestion. There is no doubt that the form of the chromosome is independent of its staining constituent.

Since the Feulgen reaction is a test only for the deoxyribose constituent of thymonucleic acid, one is not certain whether the rest of the molecule might not remain. This can be tested by observations of ultraviolet absorption. Mr. Hayashi and I have taken photographs of *Sciara* material at wavelengths 2650 and 4380. In the visible, the chromosomes are clearly made out both before and after nuclease treatment. But in the ultraviolet, after nuclease treatment, the chromosomes absorb no more strongly

than the cytoplasm and are hardly discernible. It is clear that "nuclease" removes both the sugar and the basic components of nucleic acid from the chromosomes.

It is now possible to be more precise as to the nature of the nuclease action. Nucleases are of three classes: 1) polynucleotidases, which break down highly polymerized polynucleotides to smaller units; 2) nucleotidases, which are phosphomonoesterases, and split phosphoric acid from nucleotides; and 3) nucleotidases, which separate the carbohydrate and the basic components of nucleotides.

Since our "nuclease" removes both the base and the sugar the point of action must be either the linkage of sugar to phosphoric acid, or else some linkage which connects the whole nucleotide to the protein, which linkage might be either through the phosphoric acid or through the base at the other end. The former possibility may be tested, since the properties of phosphatases are well known, and enzymes like intestinal phosphatase actively dephosphorylate nucleotides. It is well known that phosphatase action is very reversible and is, therefore, inhibited by inorganic phosphate. In most of our experiments, we have used intestinal phosphatase (beef) and studied its action in the presence and absence of phosphate. Phosphate very effectively inhibits the splitting of the nucleic acid from the chromosomes. Therefore, it may be concluded that the "nuclease" action we have been describing is a phosphatase action, and that the nucleic acid is attached to the rest of the chromosome through its phosphoric acid.

The study of phosphatase action on chromosomes also gives some insight into the state of aggregation of the nucleic acid in the chromosomes. Levene and co-workers (1939) have found that the rate of dephosphorylation of thymonucleic acid by intestinal phosphatase decreased as the molecular weight increased. They also found in the intestinal extract an enzyme which they called a nucleodepolymerase, which reduces large polynucleotide molecules to smaller ones preparatory to dephosphorylation. The depolymerase may be separated from the phosphatase by adsorption on aluminum hydroxide.

I have prepared intestinal phosphatase presumably free from the depolymerase in this way, and find that it is still effective in removing nucleic acid from salivary chromosomes. Although these experiments have not yet received adequate chemical control, the conclusion would be that the nucleic acid is not present in the form of very high molecular weight units.

Caspersson has been working on the same question, using dichroism in the ultraviolet as a measure of the extent of orientation of nucleic acid molecules along the chromosome axis. He takes issue with the conclusion of Schmidt (1939), and others, that the birefringence observed in the visible is adequate evidence of orientation in the living chromosome.

Caspersson considers this birefringence to be distinctly an artefact. Caspersson also points out that his findings do not exclude the orienting effect of nucleotide chains on polypeptide chains postulated by Astbury and others, since units as small as molecular weight 20,000 would be adequate for this.

THE ENZYMES OF CHROMOSOMES

The nucleus has often been postulated to be the center of the metabolic activity of the cell. So far as energy turnover is concerned, all the evidence on this point has been negative (Brachet, 1938). The nucleus seems to be occupied with the process of reproduction. We should like to study the reproduction of "phenotypic" genes, but this we cannot do. However, everything in the chromosome is reproduced including such easily identifiable constituents as nucleic acid. The reproduction of nucleic acid, in the process by which a chromosome makes more chromosome, may be studied by means at our disposal.

Where the reproduction is rapid, the problem of raw material is important. Koltzoff (1938), Painter (1940), and Brachet (1940) have suggested, in the case of the egg, that the raw materials are formed during oogenesis and stored in the cytosome as ribonucleic acid or its constituents. Caspersson and Schultz (1938), Brachet (1936) have found evidence of such storage not only in eggs, but also in the cytoplasm of other rapidly dividing tissues.

Recently, Dr. Ballantine and I have begun to study the enzymes that may be involved in the synthesis of new chromosome nucleic acid. The material I am about to report represents only a brief summer of exploratory work, and necessarily has many gaps.

We have studied two of the enzymes that might be involved in the synthesis of nucleic acid in the chromosome. First, we have studied phosphatase or nucleotidase. Secondly, we have studied polynucleotidase activity.

Our material was the eggs of *Arbacia*. For preparation of nucleated and enucleated fragments we used the centrifuge technique (Harvey, 1940). It

TABLE 1. EFFECT OF FERTILIZATION ON ACTIVITY OF *ARBACIA* PHOSPHATASE

Experiment	pH	mg. P/cc. eggs/hr.	
		Unfertilized	Fertilized
6/23	7.0	0.625	0.635
6/16	4.3	1.27	1.01

was found that whole *Arbacia* eggs possessed a high phosphomonoesterase activity, dephosphorylating both glycerophosphate and depolymerized nucleic acid. The phosphatase is remarkable for its very acid pH optimum. In fact, we did not go low enough on the pH scale to find an optimum.

TABLE 2. PHOSPHATASE ACTIVITY OF *ARBACIA* EGG FRAGMENTS
Substrate: 0.5 percent sodium glycerophosphate
pH: 5.3
Temperature: 25°

Cell	mg. P/cc. eggs/hr.
Whole	1.93
Nucleated half	2.10
Enucleated half	3.30

The problem of localization was attacked in two ways. First, fertilized eggs (having a diploid chromosome number) were compared with unfertilized. Secondly, the phosphatase activity of nucleated and enucleated egg fragments was compared. Tables 1 and 2 summarize these two experiments. Fertilized and unfertilized eggs possess the same activity, and there is no indication that nucleated fragments contain a store of the enzyme. If nucleotides are synthesized or broken down in the nucleic acid metabolism of these cells, the process takes place in the cytoplasm.

If the chromosome contains nucleic acid of any degree of polymerization, there should be present in the cell a polynucleotidase. The presence of such an enzyme may be measured in two ways. First, indirectly, by its effect on the rate of dephosphorylation of highly polymerized nucleic acid. We have found that *Arbacia* material acts on nucleic acid which has not previously been depolymerized, indicating the presence of the depolymerase. Secondly, depolymerization leads to a decrease in the viscosity of nucleic acid solutions. Such experiments require careful control since the viscosity of these solutions is sensitive to external factors. We used the far from ideal Hess method of viscosity determination, in order to work with small amounts of material. Since care was taken to maintain a constant pressure head, thixotrophy was not a variable.

It was first found that the whole *Arbacia* egg does contain an active depolymerizing enzyme. This is not a phosphatase since it is not affected by phosphate and works at alkaline pH values at which *Arbacia* phosphatase is inactive. We have not yet determined the complete activity-pH curve of the polynucleotidase.

Table 3 shows the effect of fertilization on the polynucleotidase activity of *Arbacia* eggs. It is clear that increase in chromosome number is accompanied by increase in enzyme activity, though the experiment does not establish a causal relation. A more conclusive experiment is given in Table 4, in which the activities of nucleated and non-nucleated fragments are compared. The answer here is very clear cut, since the non-nucleated fragments show no activity whatsoever, while the nucleated fragments are active.

In order specifically to attribute the activity to

the chromosomes, we plan to separate the fragments after the nuclear membrane has broken down, following E. B. Harvey (1940). That the activity is in the chromosomes is strongly suggested by the effect of fertilization, since here little "nuclear

TABLE 3. EFFECT OF FERTILIZATION ON ACTIVITY OF ARBACIA POLYNUCLEOTIDASE

Substrate: 5 percent sodium thymonucleate
pH: 9.0
Temperature: 25°

Experiment	Viscosity change. Centipoise/cc./hr.	
	Unfertilized	Fertilized
8/15	0.29	0.73
8/21	0.26	0.69
8/23	0.31	0.70

TABLE 4. POLYNUCLEOTIDASE ACTIVITY OF ARBACIA EGG FRAGMENTS

Substrate: 5 percent sodium thymonucleate
pH: 9.0
Temperature: 25°

Experiment	Viscosity change. centipoise/cc./hr.		
	Whole egg	Enucleated half	Nucleated half
8/13 (unfertilized)	-0.37	0.00	-0.84
8/17 (unfertilized)	-0.19	0.00	-0.476
8/19	-0.51	-0.14	-1.79

sap" is probably introduced with the sperm cell. However, activity in the nuclear membrane certainly can not be excluded.

Control experiments were carried out with heated egg extracts. The enzyme is heat-labile, which would distinguish it from the ribonuclease of Dubos (1938) and Kunitz (1940).

This experiment seems strongly to support the ideas of those who consider that much of the preparatory work in the formation of chromosomal nucleic acid takes place outside the chromosome. In fact, it would seem that the chromosome itself, if we can assume the reversibility of the polynucleotidase, begins with fully prepared nucleotides or even small polynucleotides, and organizes them into the more complex and presumably more specific polynucleotides that seem so important in gene reproduction.

An enzyme which is localized in a chromosome, and which is active in the process of chromosome reproduction, must itself reproduce! In a system like the *Arbacia* embryo the mitotic rate does not fall off during early development, and the amount of chromosome increases at an accelerating rate as

does the amount of thymonucleic acid (Brachet, 1933). Since our polynucleotidase is in the chromosomes, it must presumably reproduce, unless it is stored in sufficient quantity at the beginning of development. With such ideal material as sea urchin eggs, with which one may obtain any amount of cells all approximately in the same phase of mitosis, one may follow the exact course of the presumed reproduction. I have carried out some experiments along this line, but the results have not been uniform. In a number of cases there has seemed to be a sharp increase in enzyme activity during early prophase. However, certain problems of technique must be solved in carrying this work further. It is not going to be easy to assure adequate contact between such a localized enzyme and an added substrate. The rise during prophase might merely indicate the greater ease of contact between chromosome and nucleic acid after breakdown of the nuclear membrane. I mention this project at some length only because it seems to promise such a direct attack on a fundamental problem.

SUMMARY

No attempt has been made to draw a diagram of the molecular organization of a chromosome. Possibly such a diagram will be a contribution of this Symposium. But the following facts have been brought out.

1) The salivary chromosome, and, very likely, the plant chromosome, seems to be composed of a continuous framework and a matrix which occupies a considerable volume.

2) The matrix seems to be composed of protein containing many acidic groups.

3) The continuous skeleton seems to be composed of a histone-like protein.

4) It is possible that the chromatic bands also contain a protamine-like substance to which the nucleic acid is attached.

5) The behavior of the chromosome toward enzymes parallels the behavior of artificial nucleoprotein fibers, suggesting a fibrous organization for the chromosome "skeleton."

6) Proteins organized into fibres are not visibly digested by intracellular proteases. It is suggested that the system is favorable to protein synthesis.

7) A nucleoprotein whose properties resemble those of histone has been separated from plant material.

8) Nucleic acid is attached to the protein part of the chromosome through its phosphoric acid residues.

9) Removal of nucleic acid does not affect the continuity of the chromosome.

10) The nucleic acid of the salivary chromosome is probably not in a highly polymerized form.

11) In *Arbacia* eggs, an active phosphatase (nu-

cleotidase) has been found. It is not localized in the nucleus.

12) Arbacia polynucleotidase is strictly localized in the nucleus. The sperm nucleus brings a portion of this enzyme with it when fertilization takes place.

13) An investigation of the reproduction of the polynucleotidase has been begun.

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DISCUSSION

GREENSTEIN: The resistance of native protein fibers to enzymes is illustrated by experiments on wool.

MAZIA: Is this case not usually attributed to the abundance of sulphur as disulphide?

MELAMPY: Wool fiber is digested by the clothes moth larva.

HOLLAENDER: Does what Mazia has said about the low polymerization of nucleic acid apply only to the salivaries?

MAZIA: Yes, I have worked only with the salivaries and could not generalize.

SCHULTZ: I wonder whether Caspersson's argument for birefringence as an artefact could not apply even to sperm, since in sperm there is so much nucleic acid in so little space.

GREENSTEIN: The degree of polymerization in nucleic acid depends on the solvent and is at a maximum in water. In sperm, a lower concentration of protein is present, and hence a higher degree of polymerization may be assumed to be present than in chromosomes where the proportion of protein is greater.

CHILD: Have you measured the polynucleotidase activity of sperm?

MAZIA: Not successfully. The problem of breaking down sperm was too difficult.

MIRSKY: I was interested to hear that the fibers formed by rolling up films of nucleohistone and egg albumin are not digested by cathepsin. Some native proteins are either not digested at all or digested very slowly by certain proteolytic enzymes whereas the same proteins are rapidly digested when they are denatured. It has been suggested that denaturation renders a protein digestible because, as a result of the unfolding process that takes place during denaturation, some peptide bonds which were inaccessible to the enzyme in the folded (or native) state become accessible. When a protein film is folded up to form a fiber, certain peptide bonds may become inaccessible to cathepsin much as they are in a native protein. This possibility can be examined experimentally by removing a portion of the nucleohistone film with a wire loop and placing it while still unfolded in a solution of cathepsin.

MAZIA: Histones when prepared as fibers are not soluble, but it is difficult to separate the nucleic acid entirely and therefore to be sure that we have unconjugated histone. No protein is soluble if prepared as fibers in the way I described.

SCHULTZ: The rate of cell division decreases as division proceeds; this can be interpreted through Brachet's data as related to using up the ribose nucleic acids in the cytoplasm.

MAZIA: If the decrease in the rate of division meant that the enzyme were "diluted" in the course of development, this would invalidate the assumption that it reproduces itself.

SCHULTZ: More likely the substrate is being used up.

MULLER: I doubt that sperm bodily brings in the enzyme but suggest that it brings in the possibility of enzyme production.

MAZIA: As said above, I have not been able to study sperm directly. Only by doing this could one test Muller's suggestion directly.

AN EXPERIMENTAL STUDY OF SALIVARY CHROMOSOMES

THEOPHILUS S. PAINTER

It is unnecessary to point out to this group that in formulating ideas about the physical and chemical nature of the gene, salivary gland chromosomes have and will, I think, occupy the foreground of our attention for some time to come, first because they are the largest and most readily accessible chromosomes that we know and second, and most important, because the bands have been shown directly or indirectly to represent the genes. This being true, it is obvious that a thorough understanding of salivary chromosome structure is a matter of transcending importance for us all. More specifically we want to know what it is we are seeing in our microscopes, how the visible structures are related to the images we have of mitotic and meiotic chromosomes and, finally, can any light be gained on the structure of the enormous lampbrush chromosomes commonly found in vertebrate eggs?

Our present ideas of salivary chromosome structure are largely based on observations made on normal or untreated elements of various species of Diptera. There has been a tacit assumption, a holdover from the "permanent spireme" concept, that salivary chromosomes are morphologically static, and so we have gone over our slides, made photomicrographs or drawings of chromosomes which show certain features most clearly, and then formulated concepts which are biased to the extent that we have centered our attention on certain stages of a complicated cycle of changes, in one or two species of animals. As biologists, we are all aware of the dangers of this procedure. As an example, a few years ago a good deal of emphasis was placed on the fact that, in certain species, the salivary gland chromosomes are not visible in normal unaltered living nuclei. But it was soon found out, by Buck and Boche (1938), that in some species the bands are visible *in vivo* and in other species they are not.

At the present time there are three interpretations of salivary chromosome structure, all based on essentially the same morphological picture. These are the alveolar concept, sponsored by Metz and his students, the multiple-thread or polytene concept, and a modification of the polytene concept advanced by Calvin, Kodani and Goldschmidt (1940) to account for the "lampbrush" and "ladder" images which they obtain when the salivary chromosomes are treated with highly alkaline solutions.

Since Professor Metz has presented his evidence and ideas in an earlier paper, it is perhaps appropriate for me to outline the concept of salivary chromosome structure which I have gained from a study of several species of *Drosophila* and of *Simulium virgatum*, so that my terms will be clear

and you will understand the theoretical background which, doubtless, colors the interpretations which I will give for the experiments to be reported here. Briefly stated, I look upon salivary chromosomes as multiple-thread or polytene structures with the qualification that the visible chromomeres and their longitudinal connecting strands are not single gene strings or simple chromonemata but compounds, in varying degrees, of these. Bands are made up of chromomeres closely appressed, but a single chromomere must be regarded as compound in the sense that it is made up of a number of homologous ultimate chromomeres, none of which singly could probably be seen. In other words, the visible single chromomeres may be compared to a package of cigarettes and the connecting strands are comparable to cables rather than to single wires.

This polytene concept, based primarily on direct observational data, is supported by two independent lines of evidence. At the beginning of their development salivary chromosomes are made up of four chromatids. No one questions this fact. These chromatids are chromomeric in structure, that is, are made up of chromomeres which are connected together by strands. After synapsis the compound chromosome grows in diameter and in length. Accompanying this there is a slow increase in the number of visible longitudinal elements from 4, 8, to 16 or more but most of the increase in diameter is due to a growth in the size of the visible chromomeres. The overall extension is without doubt due, in part at least, to an uncoiling process which, as Koller (1935) has pointed out, explains the relational coiling of the two homologues. This uncoiling also may explain why, as the chromosomes grow in diameter and in length, we see a more and more complex pattern of bands. The growth in the size of the chromomeres is presumably due to a reduplication without a visible division of its parts. This reduplication without division accounts for the great increase in nuclear volume, often of the order of 512, 1024 or more times the initial volume. I think that during chromosome growth the gene string has actually been reduplicated the approximate number of times the nuclear volume indicates, but the physical separation of the parts has lagged.

A second line of evidence is even more convincing. We all know that during larval growth in insects the increase in the size of an organ is often due to an increase in the size of its contained cells and their nuclei, rather than by mitosis. It has been further repeatedly demonstrated that this increase in cell and nuclear size is accompanied by a division of the chromosomes so that these large nuclei are

actually polyploid to a high degree. There is no reason to suppose that salivary cells are any exception to this rule. They undergo division but the parts hang together for reasons which will appear later in this discussion.

I am confident that the picture of salivary chromosome structure outlined is essentially correct. It all makes sense. On the other hand, I have been keenly aware that there are morphological facts and experimental evidence which do not fit into this simple picture. If salivary chromosomes are bundles of chromatids it should be simple to separate the parts by microdissection methods. Buck (1938) has shown this not to be the case; they are tough, fairly solid structures. Anyone who has studied these chromosomes intensively will have seen places where it looks as if the chromosome had broken and material had poured out. In the *Drosophila* species which I have studied, and in *S. virgatum*, the longitudinal fibers predominantly are parallel or slightly oblique to the long axis of the chromosome (because the homologues are twisted about each other) but in some definite local areas, the fibers appear to form a net-work, such as Metz shows so well in his photomicrographs of *Chironomus*. These discrepancies, which I have been unable to account for up to the present, mean simply that we do not have a complete picture of salivary chromosome structure. We need to extend our knowledge and one method of gaining new information is to subject these chromosomes to controlled experimental procedures.

In general, salivary chromosomes have been exposed to reagents which are known to dissolve proteins of one sort or another. Intact glands have been dissected out in Ringer's solution, placed in the experimental solution for a specific time, then quickly fixed with 45 percent acetic acid and stained with aceto-carmine. After staining the glands are crushed to spread the material. Needless to say all solutions were well aerated before use and precautions were taken to guard against evaporation, an important matter in our dry Texas climate. Initially, several glands were treated at one time but it was soon found desirable to treat only one of the pair of glands, its mate being held in Ringer's solution. Later, the control gland is fixed on the same slide with the experimental, stained and mounted under the same cover glass. I have been using two species of Diptera, *D. hydei* and *S. virgatum*.

From the outset I have experienced difficulty not only because of the great variety in the responses found in a single gland but in getting the chromosomes from different larvae to give consistently the same qualitative responses to experimental treatment. At first this was baffling but some of the sources of variations soon became apparent, and this brings me to point out that as an experimental material salivary chromosomes present certain inherent difficulties which must be clearly recognized, by all of us in our different approaches, and taken into account if our conclusions are to have lasting value.

Salivary chromosomes are not static morphological structures, like metaphase chromosomes, but are constantly undergoing changes which involve differences in their physical and chemical make-up which, in turn, condition their responses to experimental treatments.

Every one knows that we often have to make a number of slides before we get a "good" preparation, that is, one which shows the band or other structures with great sharpness. Two larvae of the same size often give glands which differ greatly in size. Within a single gland there is generally a wide range in nuclear and in chromosome diameters. Furthermore, in two adjacent nuclei of the same size, the chromosomes react differently to the same fixative and stain. In one cell, the bands will be very sharply delineated and the spaces between bands water-clear, or fibrous. In the next cell the bands will stain lightly, as if less thymonucleic acid were present, or the chromosomes may appear turbid and the bands obscure. In a single normal gland of *S. virgatum*, for example, about 60 percent of the nuclei show the typical structure, the remaining 40 percent have chromosomes which are aberrant in various respects. We have all seen this, but as long as our interest was centered on band pattern, we selected the good chromosomes and disregarded nuclei in which the bands did not show so well. But when we expose a gland to experimental procedures, it is necessary to take all nuclei into consideration if we wish to have a complete picture. The truth is, we can never be sure of the exact size of a chromosome before treatment, or how it would have reacted to the fixative and stain. The best we can do is to keep one of a pair of glands as a control and study both normal and experimental nuclei together.

There are many reasons why we should expect chromosomes in a single salivary gland to give different responses. Certainly an important factor is the changes within chromosomes connected with the growth or reduplication process. If, as there is every reason to believe, endomitotic changes are occurring in salivary nuclei, some nuclei will be physically and chemically in the equivalent of a resting stage, others in a prophase, and so on. This carries with it changes in the degree of hydration and hence of the pH, differences in degree of extension, and in the amount of thymonucleic acid associated with the chromosomes.

A second feature, of which we know very little, is the relation between salivary chromosome form and the secretory state of the cells. And, finally, since eventually all of these salivary nuclei will undergo cytolysis, we must not overlook the possibility that some aberrant nuclei were necrotic before treatment.

EXPERIMENTS WITH ALKALINE SOLUTIONS

Early last summer Calvin, Kodani and Goldschmidt (1940) published a paper dealing with the

effects of highly alkaline solutions on the salivary chromosomes of *D. melanogaster*. The striking and aptly named "lampbrush" and "ladder" stages found among the treated chromosomes furnish, in part, the basis for a new interpretation of salivary chromosome structure which is essentially as follows. They accept the fact that the fully developed chromosome is a bundle of chromonemata but they think that the four original chromatids, of which all salivary chromosomes are composed initially, retain their integrity; the chromonemata formed later, as the chromosomes grow in diameter, are longer or more extended than the original chromatids and are draped about the latter, the points of contact being, presumably, homologous chromomeres. Nucleic acid is deposited about or between the loops and when the chromosome is placed in the alkali the nucleic acid shrinks about the center with the chromomeres leaving the loops clear. It is the latter which we see as the fibrous extensions in the "lampbrush" images. This explanation is offered not only to account for the observed effect of the alkali treatment but also to explain the structure of the true lampbrush chromosomes found in vertebrate eggs. Attention is called to the fact that in true lampbrush chromosomes there is a central axis of a single or a double chromonema with chromomeres; these, in oocytes, are extended prophase chromosomes which later contract into the metaphase elements of the first meiotic division. The loops and side branches, which extend at right angles to the central axis, just as in salivary chromosomes, are more extended chromonemata which are subsequently sloughed off and become material for the growth of the egg. In terms of old cytological terminology, the central axis is composed of idi chromatin and the side branches are trophochromatin. These conclusions have a far reaching importance for the whole question of chromosome structure.

Before describing and attempting to interpret the results of my own experiments with alkaline solutions, two features of the experimental procedure should be explained. When a living intact salivary gland is placed in a solution with a high pH, the whole gland usually undergoes violent changes in form, for a few seconds, during which most of the cell walls are destroyed and we have, almost immediately, an artificial syncytium produced. These disruptive changes generally break down the nuclear walls of the larger cells freeing the chromosomes which may lie near the edge or deep within the syncytium. In effect, the chromosomes are being exposed to different concentrations of OH ions, for chromosomes lying near the edge of the mass show more advanced stages in a given reaction sequence, and free-lying chromosomes are generally more severely affected than those lying within an intact nucleus. In view of these facts, although buffered solutions were used in many of the experiments, the actual concentrations of OH ions about the chromosomes of a single gland probably cover a rather

wide range and hence too much attention should not be given to the supposed pH, in any chemical interpretation of the results.

A standard procedure for isolating nucleoproteins is to digest tissue in a solution of NaOH and then to precipitate the nucleoprotein from the solution with strong acetic acid. Our treatment of salivary chromosomes follows this procedure, and we may therefore anticipate that on short treatments, due to the fact that the dissolved nucleoproteins have not diffused out of the chromosome, we will have two types of material, the remains of pre-existing structures and unorganized nucleoprotein material which has been thrown out of solution with the acetic acid.

I will present my data in the reaction sequences found after a given treatment, but I must emphasize that the percentage of nuclei exhibiting a certain type of reaction varies widely in different larvae.

Last summer I made up a 1/10 N solution of NaOH, treated the salivary chromosomes of *D. hydei* with it and obtained reactions similar to those described by Calvin, Kodani, and Goldschmidt, for *D. melanogaster*. I now feel sure that the pH of this solution was not 13 but much weaker, due possibly to the use of old chemical reagents. In this series of experiments there are 3 different types of responses given by the salivary chromosomes.

Type I. In old larvae some 87 percent of the nuclei show a slow progressive loss of staining capacity due, presumably, to the removal of nucleic acid about the chromomeres, making the bands appear more granular. In some nuclei there is a slight shrinkage of the bands away from the periphery of the chromosomes revealing the presence of an optically homogeneous material in which the chromonemata are embedded. Let us call this material a matrix. The banded organization persists as long as the matrix is intact. After about 50 minutes, however, the matrix appears to dissolve and its place is taken by a halo of tiny fibers which appear to radiate out into the nucleus. The remains of the bands fluff out and the nucleus becomes filled with a tangle of fibrous material. My interpretation of this sequence is this: The chromonemata are embedded in a firm matrix which holds the chromatids in place while the nucleoproteins are dissolved out. The matrix is more resistant to the alkali but it eventually dissolves, the chromosome outline disappears and tiny fibers take the place of and are derived from the material of the matrix.

Type II. In a small percentage of nuclei, there is a marked separation of the bands from the periphery of the chromosome (fig. 1) due probably to a shrinkage of the chromomeres, I think, although it might be due to a swelling of the matrix. Within the banded area we have the normal structure which persists for sometime while the nucleoproteins are being dissolved (figs. 2 and 3). Eventually the connecting fibers between the bands are dissolved and a chromatin residuum comes to lie, more or less disorganized, in the middle of the chromosome

(fig. 4). Later, if the chromatic core dissolves out first we have the empty matrix, which resembles a pollen tube or the hypha of a mold, but if the matrix is decomposed first its place is taken by tiny achromatic fibers, just as in type I.

The apparent explanation of the type II sequence is this: the matrix is in a different physical and chemical state than in type I; the chromonemata are held less firmly in place so the chromomeres tend to coalesce, as in many other experiments, into larger aggregates. In other respects, the two sequences run parallel and we note that the radiating fibers do not appear until the matrix begins to dissolve.

Type III. In some nuclei there is a rapid disruption of normal chromosome structure. The chromomeres show a marked tendency to coalesce into irregular masses, often involving several different bands; the chromatin seems very resistant to the alkali, and numerous tiny fibers appear radiating out, in part at least, from the individual chromomeres, giving a "lampbrush" image. In this type, the matrix is either absent as a discrete layer (fig. 8), or it is laid down about the chromonemata in a very broad zone, often with ill-defined and coalescing boundaries (figs. 5, 6, and 7). A heavy band may contract into a single lump or separate into 2 or 4 clusters, the line of separation being between homologues, or the four original chromatids. The longitudinal fibers, being intact, are drawn together into 1, 2 or 4 compact bundles; this crowds the smaller chromomeres and makes the fibers appear granular and deeply staining. Many other changes may be noted all of which can be readily understood as resulting from the coalescing of the chromomeres and its sequelae. These chromosomes are very resistant to alkali and still stain deeply long after the walls of the matrix have disappeared (fig. 8).

The explanation which I offer for the sequence type III is this: the matrix either swells and undergoes disintegration rapidly, or it is in a very diffuse state in the first place. The appearance of the fibers radiating out into the matrix, like types I and II, is an indication of the destruction of this material as a discrete structure. Why the chromatin is much more resistant to the alkali in these chromosomes, I am unable to explain.

SODIUM HYDROXIDE IN ISOTONIC SALT SOLUTIONS

In this set of experiments the salivary glands were dissected out in an isotonic salt solution (0.75% NaCl) to which enough NaOH was added to give a concentration of 1/100 N for the latter. The solution, which has a pH in the neighborhood of 12, causes a disruption of the cell and many nuclear walls in the gland and seems to render the chromosomes very soft and ductile.

The first effect, exhibited more or less in all nuclei exposed to this solution for one minute, is a tendency for the material within the natural segments

to round up into pellet-like masses separated by clear spaces (figs. 10 to 13). Almost simultaneously with this internal segmentation, the chromosomes begin to show reaction sequences.

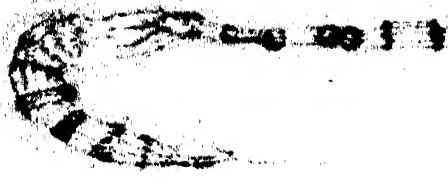
1) Most of the nuclei, in old larvae, show chromosomes with this type of behavior. The chromosomes seem dense in structure with sharply incised outlines. After nodule formation the chromosomes become pronouncedly fibrous (figs. 10, 11), then the bands disappear and the interior of the nodules stain deeply and sometimes seem alveolar (fig. 11). Next the fibers disappear, the intensity of the stain diminishes and the interior of the chromosome is filled with a finely granular material (figs. 13, 14). Sometimes (figs. 14, 15) the center of the nodule contains deeply staining large granules. Later these chromosomes swell, the outlines disappear and the nucleus becomes filled with an indistinct granular and somewhat fibrous material.

My interpretation of this sequence is the following. As in the other experiments we are dealing with chromosomes which are dense in structure. The chromonemata are held firmly by the matrix. The fibers and bands are dissolved rapidly, and the deeply stained granular material (figs. 11 to 15) represents reprecipitated nucleoproteins. On longer treatment the dissolved materials diffuse out, hence the loss of staining reaction. Eventually the matrix swells, then is dissolved freeing the materials which are left in the chromosome.

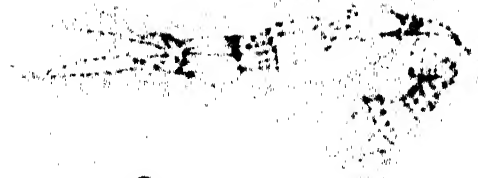
2) In some nuclei, with very broad chromosomes, the structure seems very loose although I have been unable to detect any sign of the matrix extending beyond the chromonemata. After the nodule formation, the fibers are extremely prominent (figs. 16, 17) and deposited irregularly about these fibers are minute lightly staining granules which are not visible in untreated chromosomes. The fibers rapidly dissolve out leaving the chromatic area separated (figs. 18, 19), so that the whole nucleus is filled with disconnected chromatic islands. For a few minutes the bands can be recognized, but very quickly the islands become hazy fibrous masses. All steps of disintegration can be seen in a gland treated five minutes.

My interpretation of this reaction sequence is this: Either the matrix is absent or too diffuse to be detected. The alkali dissolves out first the tenuous protein fibers between the bands, leaving disconnected the more resistant chromatic bands. The latter soon are dissolved and the unorganized fibrous masses observed represent the reprecipitated nucleoproteins derived from the bands.

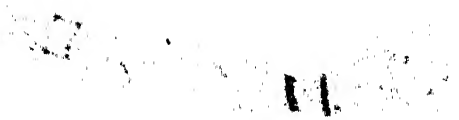
3) The first two reaction sequences comprise over 95 percent of the nuclei, in my slides. Here and there, however, nuclei are found in which the chromatin contracts into dense masses (fig. 20). The fibers are very heavy; it looks as if the chromatin was squeezed out on them. This type of chromosome is resistant to the action of NaOH and persists, with little change, for some time.



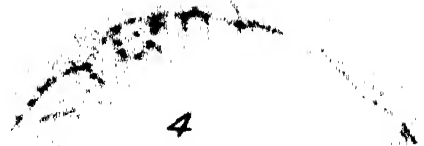
1



2



3



4



5



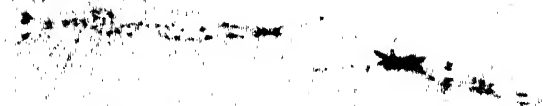
6



7



8



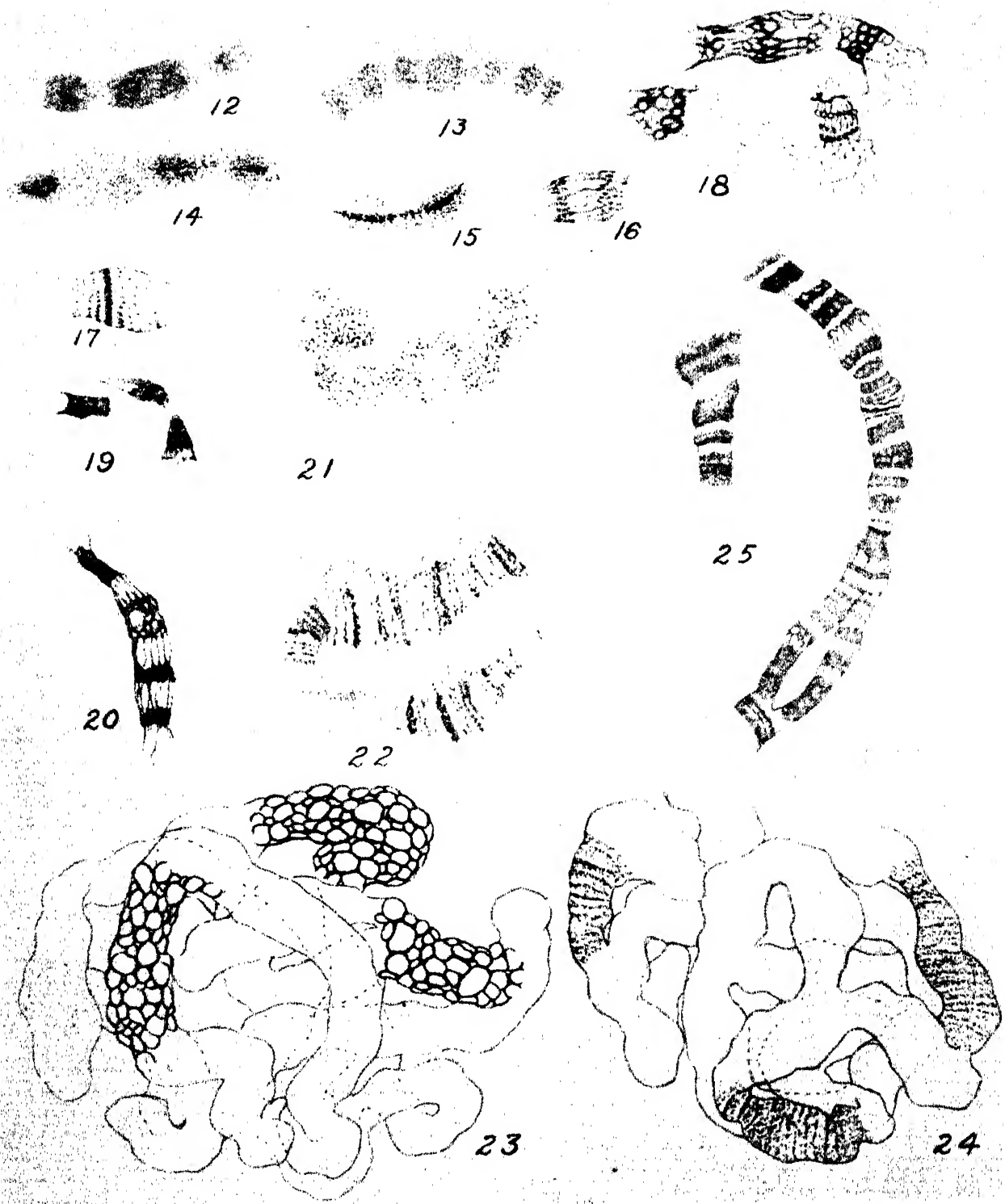
9



10



11



I do not know why, in some chromosomes, the NaOH does not destroy the chromatin, but the type of nucleus just described, in other experiments, gives rise to the striking ladder and lampbrush images.

The two sets of experiments just reported give us a good idea of the nature of the changes produced in salivary chromosomes by NaOH. Other experiments with this reagent on both *D. hydei* and *S. virgatum* can be briefly summarized.

In a buffered solution of pH 11, a vast majority of the chromosomes, in *D. hydei* show the following reaction sequence. First, there is a marked tendency for bands to associate in nodules leaving clearer fibrous spaces in between, much as in figures 10 to 13. The bands now rapidly disappear and simultaneously the nodules stain deeply but are more opaque, and the interband areas are coarsely fibrous. Next the whole chromosome takes on a fibrous structure, progressively the staining capacity decreases and soon we have an empty core or cylinder of material which I interpret as the matrix. This material begins to wrinkle in longitudinal and transverse folds, and swells and dissolves, without the appearance of any radiating fibers.

With a 1/10 N unbuffered solution of NaOH the following behavior is observed in *D. hydei*. The chromosomes shrink and even after 10 seconds treatment, a vast majority of the chromosomes show no bands. Subsequently we find about the same sequence given for the solution of pH 11 except that the changes take place more rapidly. With this treatment I have found, in some preparations, nuclei which respond differently. In some chromosomes there appear deeply staining granules, much like that shown in Figures 14 and 15. In other nuclei, the chromosome outline is extremely jagged, the bands which are more or less fused, protrude while the interband spaces are shrunken. On longer treatment such chromosomes would probably give "lampbrush" or "ladder" images.

In *Simulium* chromosomes, a buffered pH 11 solution of NaOH produces no noticeable effect up to about 15 minutes, in my experiments. After 15 minutes' exposure some of the chromosomes disintegrate by the following steps. The chromosomes become opaque, though at first the bands can be seen. Next the chromosome outline disappears, allowing the bands to spread laterally. These soon disappear and the nucleus is filled with an unorganized fibrous mass.

Apparently, in such nuclei, the matrix is first affected by the treatment, causing the chromosome outline to disappear. Later the chromonemata are dissolved by the NaOH.

In a buffered solution of pH 12 *Simulium* chromosomes are violently affected. After one-minute treatment, the nuclei can be divided into three reaction sequences. In about one third of the cells, the chromosomes, while somewhat opaque looking, show normal banding. In another third, all bands are gone and the interior of the chromosome is filled

with granules, but the chromosome outline is very sharp. In the remaining nuclei the chromosomes soon lose their outlines, are granular at first (fig. 21) and then vaguely fibrous, as in the pH 11 treatment.

In a 1/10 N solution of NaOH, even after 20 seconds exposure, most of the chromosomes show as empty cores of translucent material. This wrinkles, swells and dissolves as I have explained for other experiments.

TREATMENT WITH HYPERTONIC SODIUM CHLORIDE SOLUTION

The experiments with sodium hydroxide indicate that the chromonemata of salivary chromosomes often appear to be embedded in a material which holds the former together and does not react as rapidly to the alkali as the chromatin and other visible elements. Of course, the question of what is the character of this matrix arises at once and a possible lead was suggested by the recent work of Siganaga (1940) who showed that when the metaphase chromosomes of *Tradescantia reflexa* were treated for 10 to 15 minutes with a 0.4 N solution of NaCl, the chromonemata uncoiled and reverted to a state simulating the resting nucleus. The explanation given was that the NaCl causes the matrix in which the chromonemata were embedded to swell. It is well known, of course, that several different kinds of proteins are soluble in dilute salt solution.

The experimental procedure consisted of immersing one of a pair of glands (its mate being held in Ringer's solution during the treatment) in a 0.4 N solution of NaCl (2.34%) for from one to 28 minutes. Subsequently the treated and control glands were preserved and stained on the same slide.

As we should expect, such a strongly hypertonic solution causes much shrinkage in the salivary gland cells and in the chromosomes, which draw away from the wall and lie balled-up in the middle of the nucleus. Most remarkable and unexpected, however, are the changes which occur in the chromosomes themselves. After short treatments one commonly finds two very distinctive types of chromosomes which we may term "alveolar" and "granular."

Typical alveolar chromosomes are shown in Figure 23. The cylindrical and sharply delimited chromosomes are like sacks filled with clear bubbles of various sizes. Between the bubbles or alveoli is deeply staining material, which with Feulgen's test proves to be chromatin. In more advanced stages no bands can be recognized and after prolonged treatment the borders of chromosomes tend to fuse and the contents flow together. Even after 28 minutes treatment, I have seen no evidence of swelling or a break down of chromosomes of this type.

The granular type is shown in Figure 24. There are usually no bands visible or at most only faint indications of them. The characteristic feature is that the whole interior of the chromosome is filled with chromatic granules which give a positive reaction to Feulgen's stain. It looks as if the chromatin,

which ordinarily surrounds the chromomeres, becomes detached and scattered through the interior of the chromosomes as the bands are destroyed. After 10 minutes or more treatment, many of the granular chromosomes show evidence of disintegration, as I will describe presently.

The proportion in which granular and alveolar chromosomes are found in different glands is quite variable. In one gland, for example, treated for 28 minutes, 40 nuclei show typical alveolar chromosomes with little or no banding, 63 nuclei have granular chromosomes about half of which are disintegrating and eight nuclei show chromosomes which are alveolar at some points and granular at others. In another gland treated for four minutes, some 65 percent of the nuclei show chromosomes of the alveolar type. I have the impression, however, that the granular type more commonly predominates.

The process by which the granular chromosomes undergo disintegration is quite clear and several stages may be seen in a single nucleus. The shrunken chromosomes begin to increase in diameter which spreads the granular and irregular fibrous content of the chromosome. Eventually the boundary of the chromosome breaks down and the whole nucleus becomes filled with what might be described as a chromatic reticulum.

Since a 2.34 percent solution of NaCl is very hypertonic (0.75% is isotonic) it is interesting to note what happens when salivary chromosomes are treated with a sugar solution with about the same osmotic strength. Examination of glands treated with a 21.9 percent solution of cane sugar show that aside from a uniform shrinkage of the cell, its nucleus and the chromosomes, little change occurs except a tendency for the chromatic bands to run together (fig. 25).

I shall attempt no further chemical interpretation of my findings beyond that already given. Physical and chemical agents have been applied in order to induce qualitative changes in the chromosomes which might throw some new light on their fundamental structure. Once such an agent is found, a more refined technique can be developed to give us exact quantitative data.

From the standpoint of cytology the experiments here reported are illuminating not only in allowing us to understand some of the properties of salivary chromosomes which have been obscure heretofore but also in extending our knowledge of chromosomes in general. Here I will discuss some of the more interesting aspects.

One of the fundamental problems in cytogenetics is the correlation between salivary, meiotic and mitotic chromosomes. In a general way we all know that we have here different degrees in the extension of what is essentially a chromomeric thread. Specifically, are the chromomeres seen in the pachytene stage identical with those which we see in salivary chromosomes? Unfortunately, comparable

data are not available. We know that in some of the lilies there are about a total of 2500 ultimate chromomeres; in *D. melanogaster* the number is possibly four times as large, and I have always felt it was probable that more chromomeres are present at pachytene than can be seen. In this connection it is interesting to note that in nearly all of the experiments, there is a marked tendency for the bands to contract together giving often a nodular appearance to the chromosomes. There seems to be a definite organization back of the contraction for I have repeatedly noted that when two homologues are unpaired, the contraction pattern is the same in each chromosome. Are these nodules steps in the formation of compound chromomeres such as those of mitosis and possibly those of meiosis as well?

An outstanding feature of all these experiments is that in any single salivary gland the chromosomes usually give three distinct reaction sequences to a single reagent. Slight differences in the local concentration of OH ions might be expected to give different stages in a reaction sequence, as we find, indeed, in every preparation, but when the chromosomes in one nucleus go to pieces in a few seconds and in another they resist the reagent for many minutes, it is clear that in these two nuclei the chromosomes were different in the first place. I think that the causes of these differences can be traced to growth cycles and the various phases of the secretory process going on in salivary cells. Of the latter we know very little except that in gland cells the chromatic reticulum is usually heavier than in resting cells. On the other hand, intranuclear division cycles would fully account for most of the phenomena which we have observed.

In normal mitosis, aside from coiling, the chromosomes undergo three known sets of changes which affect their physical condition and, presumably, would greatly modify their responses to chemical agents. 1) The water content of chromosomes, and the associated changes in pH, varies, the interphase elements being most hydrated, the metaphase least (Kuwada, 1937). 2) Recent work by Caspersson and Schultz (1938) has shown that the total nucleic acid content of the nucleus changes with the mitotic phase, the resting nucleus being poor and the prophase relatively rich in this material. 3) Although there has been sharp disagreement on this point, many cytologists have long believed that, beginning in early prophase, material, usually called a matrix, is deposited about the coiling chromonemata and persists as a protective coat until late telophase when it dissolves and forms or merges with the nuclear sap.

The experiments here reported furnish definite and critical evidence for the presence of matrix material in which the structural elements of salivary chromosomes are embedded. It is this material which holds the chromatids together and gives salivary chromosomes their mechanical strength and toughness. But the character of the matrix differs

in different nuclei, as we should expect if endomitotic changes are taking place. In some nuclei it either is not present as a formed structure or else it is very rapidly destroyed. When this happens the chromosome loses its outline and the chromonemata, or their remains, spread about the nucleus. More usually the matrix is extremely resistant to alkali. In any event, often accompanying the destruction of the matrix is the appearance of tiny fibers which radiate out from the center of the chromosome. This, it appears, is the real explanation for the fibers seen by Calvin, Kodani and Goldschmidt in their lampbrush images.

If a matrix exists about chromosomes of other types, it would play a role in the mechanics of coiling, as Kuwada and his students, and Huskins rightly maintain, and it would give us an explanation for many facts which do not now fit in with our cytogenetic concepts. It explains, for example, the anastomosing processes generally observed (and attributed to poor fixation) between chromosomes in the telophase-prophase range of changes. And it adequately explains the nature of the radiating fibers associated with true lampbrush chromosomes in vertebrate eggs. In the toad egg, these lateral processes do not stain with Feulgen's reagent, but when one examines such chromosomes with sharply oblique light he sees that the lateral processes are extensions of a transparent coat which surrounds the Feulgen positive chromomeres. It is this matrix which gives lampbrush chromosomes their strength and allows them to be stretched more than half a millimeter (Duryee, 1937).

In an earlier paper (Painter, 1940) I expressed the opinion that salivary chromosomes were surrounded by a pellicle or a perinema. I am now rather inclined to the simpler explanation that the pellicle is not a separate structure but the boundary of the matrix.

Finally, throughout these experiments I have been impressed with the fibrous character of salivary chromosomes. In my preparations I see little evidence for an alveolar structure. You start with chromomeric chromosomes, initially, and you end with a bundle of chromomeric chromosomes held together by a matrix.

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DISCUSSION

METZ: I would like to make the following comments on the morphological features of Painter's material. 1) I do not agree that the conditions shown, following the alkali treatment used, represent real conditions in the chromosomes; they show strong evidence of distortion and disruption of the structure. 2) I do not think that chromomeres are without significance; but on the other hand I do not think they have the peculiar significance which Dr. Painter attributes to them here. The question is: do the original chromonemata multiply until they form bundles of more than a hundred each; and then do these bundles have the power of accurate division into equal daughter bundles; do these then undergo internal multiplication, followed by accurate division again, and so on; and do individual loci in the chromosome have their own special rates of division of the bundles, to account for the widely different numbers of "chromomeres" at different loci? These are all special attributes which chromonemata are not known to possess, and which the ancestors of these chromonemata almost certainly never manifest. I think we should be very cautious about assuming, without convincing evidence, that chromonemata possess such potentialities. Such an assumption is very different from merely assuming that the entire chromosome is a huge multiple of submicroscopic chromonemata and that achromatic droplets or vesicles within it separate these chromonemata into clusters of various numbers and sizes in different regions.

STADLER: Over how long a period does growth of the salivary cells take place?

PAINTER: There are nuclei of all sizes in any gland in *Simulium*.

STADLER: Could not crucial figures be obtained from derangements induced by irradiation during the growth period?

METZ: The difficulty is to find any practicable way to do this. It has been tried, but no one has yet succeeded.

STADLER: What is known of the structure of the salivary gland chromosomes during development?

SONNENBLICK: In the eight-hour embryo, the salivary glands contain about 125 nuclei each, and counts of nuclei show no further divisions. Because of the small size of the nucleus, about three micra long in the early stages, the structure of the chromosomes cannot be seen. In those larval tissues where polytene chromosomes occur, I have observed no mitosis after laying down of the tissue in embryonic development. It is possible that in the embryonic period, intranuclear divisions may take place.

METZ: In the development of *Sciara*, also the glands show no further divisions after they are laid down. A mosaic case shows that the gland

cells are not the lineal descendent of any one cell. Growth of larval tissue without cell division is characteristic of holometabolous insects.

BREHME: Actually, the growth of larval tissue by increase in cell size and growth of imaginal tissue by cell division during the larval period of holometabolous insects has been demonstrated for only four insects, *Culex*, *Bombyx* and *Lucilia* by Trager, and *Tribolium* by Smallman, although it is generally assumed that this type of growth is characteristic of this group.

METZ: Are there any cases where this type of growth has been found not to occur?

BREHME: No, there are not.

MAZIA: The pH variations that could occur in living nuclei might give a normal occurrence of all the pictures which Dr. Painter has obtained experimentally, if sufficient time were allowed.

MULLER: In his work on chromosome multiplication by measurement of cell size, Jacobi got a polymodal curve, although the successive peaks did not always represent a regular doubling of the volume. Hence there must be a periodic limitation of the growth process in time, otherwise the curve would not be polymodal. Wermel confirmed this periodicity by cinematophotography. He found that in a given nucleus there are considerable periods when there is no growth, and that the growth takes place in sudden steps. He used salivary glands as well as other tissues.

There is no evidence that one chromosome in a

nucleus ever reduplicates without all the others doing so, hence the regular multiples found and the possibilities of judging the amount of reduplication by fairly gross volume studies. The inference from these studies is that there are thousands of components in each mature salivary gland chromosome. From this in turn it follows that the chromonemata would be too fine to be seen, as Metz contends.

MICKEY: The lampbrush appearance might be derived from chromatic material which is not matrix.

PAINTER: The Feulgen reaction shows no fibers, only granules.

COLE: With Boche, I have made photographs of *Chironomus* chromosomes within the gland with the ultraviolet microscope using 2804 Å. A thread-like structure may be seen but it could be alveolar as well.

HUSKINS: The matrix has probably more importance than has been accorded it in the last few years. 1) It may hold double chromosomes together, as in Berger's spinach case. 2) Chiasmata are significant in holding the chromatids together, but they are not necessarily held by chiasmata in late prophase and metaphase; furthermore, chromosomes may fall apart following heat treatment although chiasmata are still present. Therefore, the chiasmata may hold the chromatids together until the matrix is formed, and then the matrix may be the agent for holding them. In the salivaries, there is great development of the matrix.

THE EVIDENCE OF THE NUCLEOPROTEIN NATURE OF THE GENE

JACK SCHULTZ

The inquiry into the nature of the gene is generally conducted either upon a purely formal plane, where the attempt is made to obtain an answer regarding certain properties that are specifically genic, from the evidence of breeding experiments or cytological analysis; or else the nature of the gene is solved incidentally, a crumb dropped from the biochemical table. It will perhaps be instructive to consider what the requirements are from the genetical experiments for a substance which is to be called the gene, and how far the substances we know anything about satisfy these requirements.

The problem was first attacked by Friedrich Miescher, and you will remember that he came to the conclusion that the substances he discovered in sperm nuclei, relatively simple substances that we now know as thymonucleic acid and protamines, might be the material basis of heredity, if the possibilities of isomerism were kept in mind. The apparent simplicity of these substances was later to be stressed (see Matthews, 1915, for example), and it was in fact used to oppose the chromosome theory of heredity. The shoe is on the other foot now, however, and our problem is to evaluate the evidence that actually the nucleoproteins of the cell nucleus do have anything to do with the self-reproducing specificities that the genetic data require.

The classical theory of the gene postulates units of heredity held together in a linear order. These units are detected as such by virtue of specific changes in the developmental process, changes which are shown to have occurred in specifically localized sections of the chromosome. The implications of this theory in terms of substances are first that a genic substance must have the ability to reproduce itself, it must have a definite and specific connection with the synthetic machinery of the cell, it must have the capacity for changing its mode of affecting synthesis and at the same time retain the power of specific self-reproduction. It must further be able to maintain linear connections with its neighbors, and finally to establish specific relations with its homologue at the meiotic stages. These are fairly platitudinous statements; the fields of modern genetics are recognizably based upon these concepts.

When we come to integrate these concepts with the chemical make-up of the nucleus the cytological study of chromosome behavior is an intermediate step. The aggregates of genes that compose the chromosomes go through a series of maneuvers which visibly show the chemical changes undergone during a division cycle, and after the establishment of a working cell, the changes correlated with

activity. The fact of these cytological changes warns us immediately that the genetical model of a chromosome map drawn from the stages of meiosis need not be interpreted in terms of a constant substance, but may be a constant set of conditions which provides for its own maintenance at reproduction. The gene, like the Roman God Janus, has two faces, one turned toward the maintenance of its own integrity, one looking toward the synthetic metabolism of the cell.

THE CONSTITUENTS OF THE NUCLEUS

The analytical data on the components of the nucleus are confessedly meager. They are restricted to a few convenient sources, and indeed there have been few innovations in this respect since the pioneer work of Miescher (1874; see the collected works, 1898). He used sperm and lymphocytes, and it is only recently that methods of separating the cell components by specific gravity have been devised and used (Behrens 1938). With the relatively few sources which we have available, it is evident, as many people have realized, that analysis of one type of nucleus may give information that is totally misleading when used to discuss the nature of the gene. In fact this fallacy is shared by the early objections to the chromosome theory, and by some of the most recent theories of chromosome structure.

The constituents of all cell nuclei are nucleic acids and proteins in varying proportions. Caspersson (1940) has recently summarized the evidence from macrochemical data concerning the nature of the proteins in the various types of nuclei. They resolve themselves into basic proteins of the protamine and histone type, with perhaps some indication of heat-coagulable protein. It is to be stressed that aside from the observations of Brachet (1938), on the enzymatic activity of isolated nuclei of frog eggs and Behrens (1939) on the arginase activity of liver nuclei, we have no analyses of composition and behavior of the actively functional interphase nucleus. Certainly the nuclei which have served as sources of chemical information—taken from pus cells, or bird blood corpuscles, or the lymphocytes of the thymus gland—do not come from cells active in synthesis.

What is of interest however in these early analyses is that each species examined had its own special type of protein, and that the nucleic acids were all of a uniform composition, as far as the methods went. For example, each species of fish sperm has its own typical protamine; and the histones obtained from other types of sperm have even more diversity. Moreover, it is by no means certain that

the substances from one species are uniform and not mixtures. Thus even our limited knowledge of the protein carries the promise that there may be the specificities that the geneticist desires within them.

The nucleic acids isolated from sperm have been conventionally treated as presenting on the other hand a rather monotonous uniformity, which has led to various hypotheses regarding their uses. All of the hypotheses usually advanced represent the nucleic acids as indices of the degree of "failure of function," a concept that may be traced to Kossel (1882). Frey-Wyssling (1938) for example, suggests that the function of the nucleic acids is to "protect" the active side chains of the protein molecule. This view has its basis in the fact that the desoxyribose nucleic acid content of the inactive nuclei is always high. Actually, it is now possible to reconcile the so-called uniformity of the nucleic acids with their possible presence in the genes in another way, which we will come to presently. It is well, however, first to question the extent of the uniformity.

Nucleic acids have been found to fall into two groups, according to the sugar they contain—the ribose nucleic acids and the desoxyribose nucleic acids (see Levene and Bass, 1931). The two groups differ also in their pyrimidine bases, the ribose nucleic acids containing uracil instead of its methylated derivative thymine. Curiously enough, in addition to these two differences there is an extensive difference in their capacity of polymerization, the desoxyribose nucleic acids forming in the native state polymers of a molecular weight of as much as 1,000,000 (Signer, Caspersson and Hammarsten, 1938). This group includes the nucleic acids found in the chromosomes and usually restricted to them.

The actual structure of a desoxyribose nucleic acid has to my knowledge been worked out in only one case, that of the nucleic acid of the thymus gland, and even here there has in recent years been some question as to the details of the linkages (see Levene and Bass, 1931 and Bredereck, 1938). When it is considered that the highly polymerized thymonucleic acid has been studied in detail from a single source, and that only recently have the ribose nucleic acids begun to be prepared in a comparably elegant manner, it is evident that the earlier conclusions can be accepted only as a first order approximation, and that much new data is necessary before we can exclude the possibility of specificities in the nucleic acids themselves.

There is moreover a purely cytological difficulty which has not received attention. It is well known that the distribution of the nucleic acid in the chromosomes is not a purely random affair. The so-called "heterochromatic" regions in some species of *Drosophila*, for example, may be estimated to be responsible for at least a quarter of the total metaphase length of the chromosomes (Heitz, 1933; Kaufmann, 1934). Now it is known that these con-

tain relatively few genes within them (Muller and Painter, 1932; Muller, Raffel, Gershenson and Prokofyeva, 1937; Heitz, 1933). Thus a preparation of nucleic acid from the *Drosophila* metaphase chromosomes would have as a major contributor only a few genes. Obviously the separation of different possible types of desoxyribose nucleic acids, some present in small concentrations, is a problem that has not yet been solved. But pending its solution, we must admit the possibility of specificities in the nucleic acids. And it is evident, as has already been implied, that the same considerations apply to the proteins.

Immunological methods have only begun to be applied to these substances, for example, the serological reactivity described from Mudd's laboratory (Lackman et al, 1941) for a group of nucleic acids, against a globulin component of various immune sera. But the state of the field may be surmised from the fact that there are no references to the protamines or histones in the index of Marrack's (1938) monograph of the chemistry of antigens and antibodies.

So far we have discussed only the possibility of specificities in the nucleoproteins, the question whether there can be enough variety in composition to account for the diversities of the specific genes. The answer is apparently that there may be, but that the demonstration is as yet not complete, a discussion to which we will return. We come next to the question whether the physical properties of the nucleoproteins are compatible with the linear arrangement of the genes, and here we are on safer ground.

With the establishment of the polypeptide structure of the protamines, the idea was advanced that in a fibrous protein structure the basic linear arrangement could be found (Koltzoff, 1928; Wrinch, 1936). More recently, it has been demonstrated that the molecules of thymonucleic acid polymerize in long chains (Signer, Caspersson and Hammarsten, 1938), and that the backbone spacing of the molecule along these chains is almost identical with that of the polypeptide chains in the proteins (Astbury and Bell, 1938). So that we have the possibility in both the prosthetic group and the protein for chain formation, and here detailed cytochemical information becomes necessary.

THE CONSTITUENTS OF THE CHROMOSOMES: CYTOCHEMICAL ANALYSIS

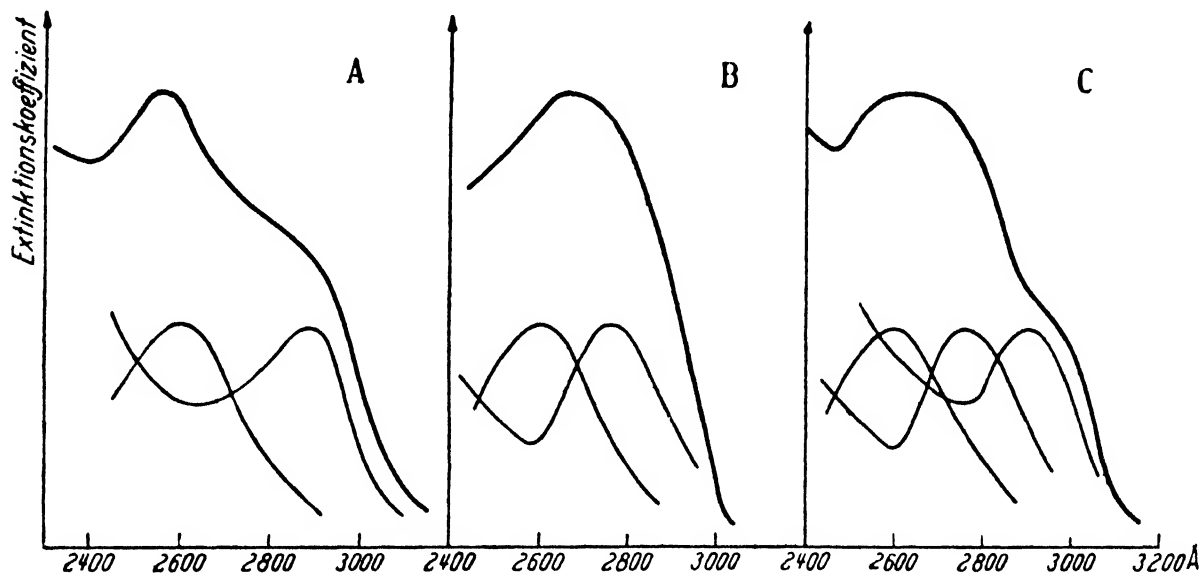
The cytochemical analysis is of some methodological interest. It involves the detection of the cellular substances by three methods: through their ability to combine with other substances that act as markers, the staining technique; their disappearance after appropriate digestion or other chemical treatment; and finally their identification by their own intrinsic properties. The nucleic acids have proved amenable to all these attacks, in that

they combine with basic dyes, are attacked by enzymes, and have a sufficiently striking absorption band in the ultraviolet region of the spectrum so that their presence can be followed by their own properties as well. The proteins are more difficult, except in their enzymatic tests. The more complex proteins, containing aromatic amino acids, do have an absorption band that has proved useful. Most important, all three methods have given the same, mutually confirmatory answer, namely that the structure of the chromosome can be described as an arrangement of nucleoprotein and protein in sequence.

The chromosomes tested by the Feulgen reaction are shown to contain sugars of the desoxyribose

light reduced by means of a calibrated rotating sector. With the newer technique, the error is reduced to about 3 percent for an extinction coefficient of .1 (Caspersson, 1940a). Further, he has been able by the use of polarized ultraviolet light to make tests of the orientation of the nucleic acid in the salivary gland chromosomes (Caspersson 1940c).

The nucleic acid absorption band, due to the nitrogenous bases contained, has a maximum at 2600; towards the long wave lengths, around 2800, lie the absorption bands of the aromatic amino acids, much weaker than those of the pyrimidines. By measurements in polarized ultraviolet light, Caspersson (1940c) has recently shown that the native thymonucleic acid exhibits a dichroism, its absorp-



TEXT FIG. 1. Types of absorption spectra shown by combinations of nucleic acids and different types of protein (after Caspersson, 1941). A, the combination of nucleic acid and a basic protein of the histone type; B, nucleic acid and a more acidic protein; C, all three substances present together. Abscissae are the wave lengths of light, ordinates extinction coefficients.

type, found only in the thymonucleic acids. That ultraviolet absorbing groups associated with these were responsible for the absorption in the chromosomes was pointed out by several workers and proof came from the ultraviolet absorption measurements made by Caspersson (1936). His early measurements were made by a photographic method, in which photographs of chromosomes, taken at different wave lengths in the ultraviolet, were measured for the amount of blackening on the plate—proportional to the absorbed light; by a proper system of calibration the absorption of any region on the photographic plate could be obtained. The error of this method was about 5-10 percent. More recently he has developed a photoelectric method, in which the image of the object is projected on to a photo cell by means of a prism, and a comparison made between the current from the image and that of a neighboring space in the preparation, with the

tion of light parallel to the long axis of the molecule being about 1.6 that of the value for absorption of light at right angles to the axis. Now the absorption of the nucleic acid containing portions of the *Drosophila* salivary gland chromosomes, portions which give the Feulgen reaction and so permit discussion in terms of the chain molecules of thymonucleic acid, is a typical nucleic acid absorption curve, with deviations interpretable on the basis of protein present. The same is true of the grasshopper metaphase chromosome. He states that photographic measurements of the absorption of bands in polarized light show no absorption differences dependent on the plane of polarization. Sperm on the other hand contain a strongly oriented nucleic acid component. The method is very sensitive, and he points out that differences far beyond those previously measured by Schmidt (1938) and Pfeiffer (1940) in *Chironomus* would have been ob-

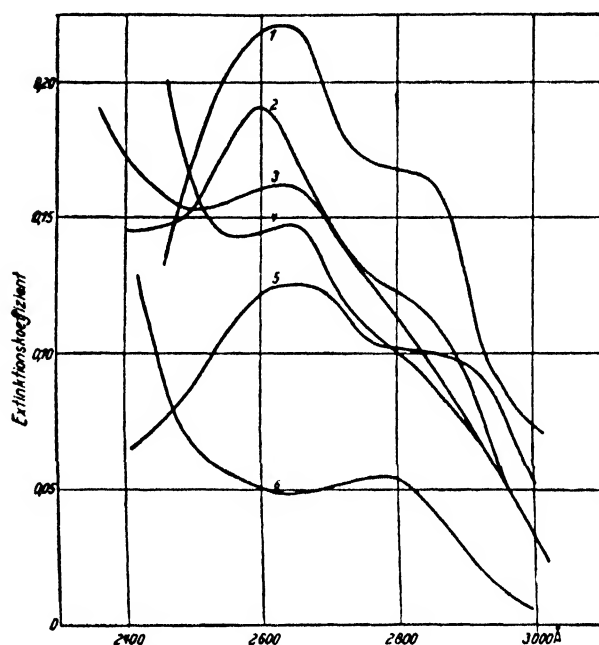
served. He attributes the discrepancy between his results and those of the other workers to their use of alcohol treated chromosomes or in Pfeiffer's case stretched chromosomes creating an artefact, in which the thymonucleic acid rods are forced into an orientation analogous to that of double refraction of flow. It seems likely from these results, and from those of the digestion experiments that the nucleic acids in the salivary gland chromosomes are present as lower polymers in which orientation need not be so precise.

The direct optical methods have also proved useful in the analysis of the proteins. Here the presence of amino-acids with aromatic nuclei can be followed, more especially tyrosine and tryptophane, which have absorption bands at 2750 and 2800. Caspersson has quite recently demonstrated (1940b) that the tyrosine band in proteins containing many basic aminoacids is shifted towards the long wave lengths, and by means of an analysis of the slopes of the absorption curves, that the presence of the different types can be detected. Thus in a protein with many acid groups, the tyrosine band appears at 2800, whereas in some basic protein such as the histone from thymus nucleoprotein, the tyrosine band appears at 2900. There is then a group of absorbing components which are summated in the absorption curve of chromosomes; the nucleic acids, tyrosine, tryptophane, the aliphatic amino-acids, and the non-specific absorption due to scattering.

It is evident that this provides an opportunity for analyzing the nature of the protein portions of the chromosomes, and thus for the extension of our information concerning not only the linear order but the specificities of the different regions as well. Actually what results is that in salivary gland chromosomes of *Drosophila melanogaster*, evidence is obtained for the presence of two and possibly three types of proteins; a protein resembling in its absorption characteristics the serum globulins, one distinctly basic in character, and in addition an absorbing component exhibiting the non-specific absorption of the aliphatic amino-acids, in other words a protamine like component. The distribution of these components in the different regions is as follows: possibly all three in the nucleoprotein bands, chiefly the "globulin" type and the "protamine" type in the usual interband spaces, and chiefly the histone type in the nucleoli and the related puff and chromocentral regions. Caspersson points out the linear structure of the chromosomes is most nearly perfect in those regions showing a high concentration of aliphatic amino-acids, and that the puffs and chromocenter, which are confused in this regard, have a low concentration of these "protamine"-like proteins. Thus the most likely substances to form the permanent linear pattern in the salivary gland chromosomes are the protamine-like proteins.

These conclusions are the result of a rather com-

plex analysis, and it is well that they are in accord with the results of the enzymatic work, both Caspersson's, and that which Dr. Mazia has discussed. Within the last month, by a combination of staining and enzymatic techniques, I have had some success in pushing the analysis a step farther. There has not as yet been the opportunity to make a detailed study, but the outlines seem clear enough to warrant report. If the salivary gland chromosomes of *Drosophila* are stained with an acetic acid orcein



TEXT FIG. 2. A comparison of absorption spectra of different parts of the salivary gland chromosomes (after Caspersson 1941). Curves 1 and 5, portions of the chromocenter, showing the nucleic acid maximum at 2600 and an "elbow" around 2900 (tyrosine in a basic protein); 2, a portion near the chromocenter; 3, a "euchromatic disc"; 4, a "heterochromatic" portion of chromosome 4; 6, an interband space.

solution, following the technique of La Cour, and then counterstained with fast green, the nucleoprotein bands are black, as Kodani has described them, and the interbands take a definite green tinge. There are however in addition bands in the chromosomes that take a green stain. It is furthermore striking that in the chromomeres at the chromocenter, and in other capsulated regions, structures already described by Bridges (1938), Bauer (1935) and Painter (1939) as being a chromatic hull with an achromatic core, the fast green stain is not found within the core: it either does not penetrate, or the material which combines with it is situated around the periphery. That the latter is the case is shown by the experiment of dissecting the glands into Ringer's solution at a temperature of about 50° and fixing after about 15 minutes. Under these circumstances a dissociation of the nucleoprotein complex

takes place, and the fast green component of the chromosomes goes off the bands which then on staining appear red from the orcein stain. This is quite striking in such a species as *D. robusta*, where the chromosomes are larger. After heat treatment the chromosomes have red bands with colorless inter-band spaces and lie surrounded by pools of green staining material. The phenomena suggest those described during fixation by Doyle and Metz in *Sciara*, and it seems probable that they were dealing with the same substances. There are thus, on the basis of their staining properties, two separable substances within the chromosome in addition to the desoxyribose nucleic acids: a substance which combines with fast green, and one which does not. I have tried various others of the common counter stains, and not as yet found any that give analogous results to the fast green. This was claimed long ago by Flemming to be specific for the nucleolar substance, and has been used quite recently by Semmens and Bhaduri (1940) in the same way, with a more precise differentiation. This would suggest as we will see an affinity for histones, and in fact smears of a histone preparation made by C. B. Metz from sea urchin sperm do stain intensely with the fast green. It seems not unlikely therefore that the fast green staining component is a protein of this type. The remarkable fact, however, is that a protamine preparation called salmiridin, sent to the Pasadena laboratory by Dr. H. C. Hagedorn, does not react with the fast green. The parallel in the staining between the portion of the chromosome which remains in the linear array after heat treatment and the polypeptide protamine is exactly what would be expected from the ultraviolet results, and from the work of Mazia and Jaeger with the nuclease from spleen.

The results of experiments with a crystalline ribonuclease (Kunitz, 1940) preparation, for which I am indebted to the generosity of Dr. M. Kunitz, add point to the story. The question of the presence of ribonucleic acids in the chromosomes in addition to the desoxyribose type seemed accessible by the use of this enzyme. Brachet (1940) had in fact already described a presumable digestion of ribonucleic acids from chromosomes in amphibia by a preparation made following the earlier technique of Dubos and Thompson (1938). Using the pyronin-methyl green technique devised by Unna, he found that substances taking the pyronin stain were removed by the enzyme from both chromosomes and cytoplasm leaving a bright methyl green stain behind in the chromosome. Accordingly I repeated the procedure, using the crystalline ribonuclease in amounts high enough so that the enzyme concentration was no longer the limiting factor for rate, but using, however, three different pH's, in which the activity was somewhat different—6.2, 7.5, 8.5. The results of the experiments showed that the fast green staining component of the chromosomes is

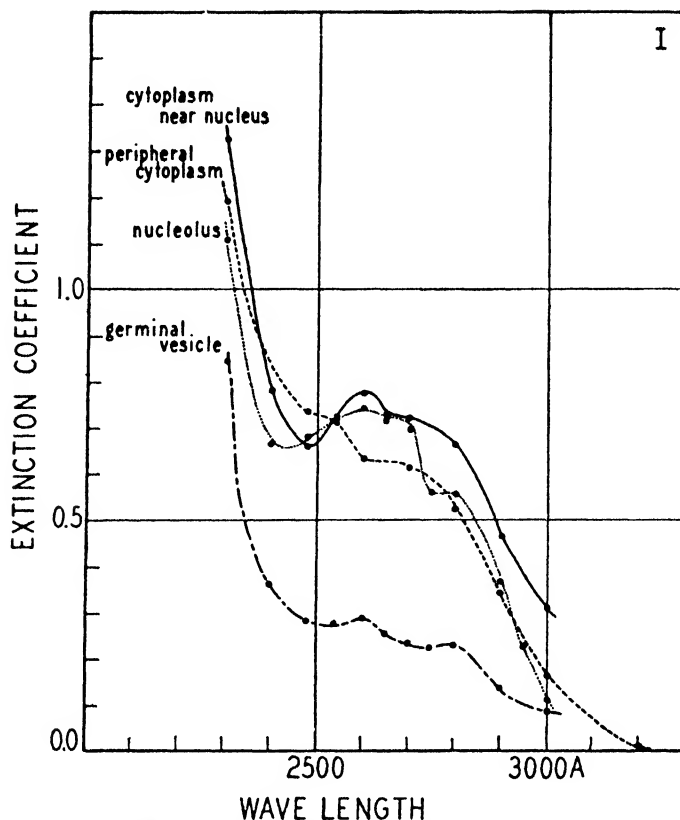
bound by a link that is attacked by the ribonuclease, and that at the greater activity the desoxyribose nucleic acid itself was lost from the chromosome, leaving in the final stages only the non-staining portion of the chromosome. With the pyronin-methyl green technique, however, even in the controls, as in the case of the plant chromosomes studied by Shinke and Shiganaga, the nucleoprotein bands were purplish red in color, and not blue-green. Treated with the enzyme, they became still redder, and attained a color more characteristic for the stain of the ribonucleic acids of the cytoplasm.

It is important that different types of nucleus react differently. In an experiment, for example, where *Drosophila* sperm and salivary glands were exposed on the same slide to the enzyme, at pH 7.5 for one hour, at 56°C., the giant chromosomes were almost completely digested, and the sperm, presumably with the higher polymer, were untouched. Similarly, experiments with thymus cells, the source for preparation of the highly polymerized thymonucleic acid, show them to be highly resistant to the enzyme, although there may be some loss of nucleic acid from the cells. If it should turn out that the linkage attacked by the enzyme exists only in the ribose nucleic acids, the results indicate that there is a "structural cement" of the ribose nucleic acids, and that inactive nuclei like the sperm, contain less of this than the active nuclei.

The digestion, except in the sperm, of the outer coat of fast green staining protein away from the chromosome is a striking effect. Such sections as the puffs are most obviously affected, but I have not yet had opportunity for a detailed study of regional specificities. The effect is not restricted to the salivary gland chromosomes; in *D. melanogaster* the mitotic chromosomes of the neural ganglia, and the chromocenters and chromatin threads of the resting cells lose the fast green stain and are red with the orcein alone after digestion, where the controls are black. From these experiments it seems possible to picture the chromosome as a protamine-like thread, with nucleoprotein at intervals, with a sheath of a more complex protein related to the nucleolus. That this is the conventional picture of a whole group of cytologists need scarcely be stated. It seems as though by the use of the ribonuclease, and controlling the reaction, we can strip off the coats successively.

THE ROLE OF NUCLEOPROTEINS IN CELLULAR SYNTHESIS: THEIR RELATION TO GENE FUNCTION

The structure of the chromosome as defined cytologically is thus in not bad agreement with the extractive analysis, and adds a topography of the specificities. I shall not have time here to enter into the evidence relating this topography to the loci of the genes. It is, however, a difficulty of such analysis that the specificities of the genes are recognized by



TEXT FIG. 3. Ultra-violet absorption spectra of different parts of an ovarian egg of the sea urchin, *Psammechinus miliaris* (from Caspersson and Schultz, 1940). It is evident that the cytoplasm near the nucleus shows a more marked band at 2600 Å. U. than does the peripheral cytoplasm. The suggestion (see Claude, this symposium) that the concentration of nucleo-protein around the nuclear membrane is due to an aggregation of mitochondria in this region does not seem to be tenable for this form, since in the sea urchin (Wilson, 1926, *Amer. Nat.* 60; p. 114) the distribution appears to be uniform, or there may even be a peripheral concentration of the mitochondria.

what they do, and the knowledge, for example, that there are different proteins in the chromosome helps only to a limited extent. The genes set the pattern of synthesis, and it is only by the analysis of that pattern that we can hope to determine the meaning of their specificities. Here again as a result of cytochemical analysis, we find evidence pointing to the nucleoproteins as playing a dominant role this time in the synthetic processes of the cell.

Evidently the role of the genes in the cytoplasmic syntheses concerns the nucleo-cytoplasmic relationships, and here the differentiation into desoxyribose and ribose nucleic acids is of great help. It turns out that the nucleic acids present in the cytoplasm are of the ribose type, and what is most important, that in cells which are functionally active or which are dividing rapidly, the concentration of the ribose nucleic acids is high. This is of course immediately

FIG. 1 A photograph, taken at 2570 Å in the ultraviolet microscope of cells from the mid-gut of a third instar *D. melanogaster* larva. The functional larval gut cell absorbs only slightly, while the small imaginal disc (embryonic) cells are highly absorbing. $\times 700$

FIG. 2 Ultraviolet photograph (2570 Å) of a section through an ovarian nurse cell. Around the nuclear membrane is an evident region of high absorption. $\times 1220$

FIGS. 3 and 4 Salivary gland chromosomes from a translocation between the X and fourth chromosomes of *D. melanogaster*, showing the translocated and normal bands (brackets) side by side. These are from XX females, raised at 16°C; the bands translocated to heterochromatin show a darkening and the assumption of a heterochromatic structure. The photographs are taken in the ultraviolet micro-

scope (ocular 20 \times , objective 1.66, λ 2570 Å). $\times 4880$

FIG. 5 The same translocation in the XXY female; the bands next to heterochromatin now approach the normal bands in character; those in the section of X translocated to the chromocenter still are darker than normal. Comparable sections are indicated by similar brackets. $\times 2400$

FIG. 6 Ultraviolet photograph (2570 Å) of the ovary of an XXY female of *D. melanogaster*. The arrow points to a nurse cell in whose nucleus the highly absorbing heterochromatic Y chromosomes can be seen. By the study of these in the nurse cells of different sizes it is found that their multiplication proceeds at a slower rate than the euchromatic chromosomes. $\times 350$

(The foregoing photographs are from joint work with Dr. T. Caspersson.)

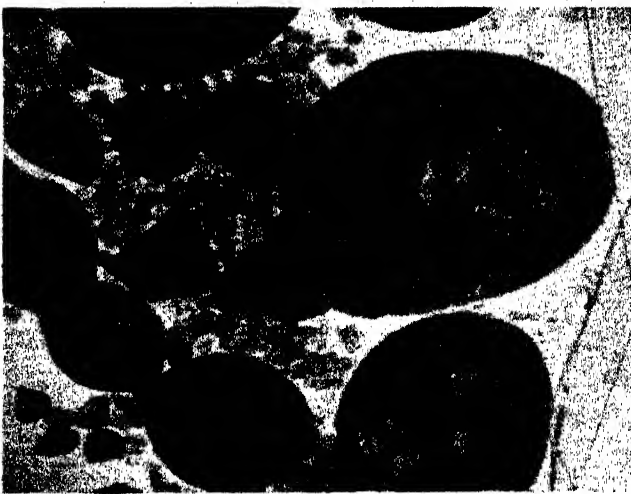
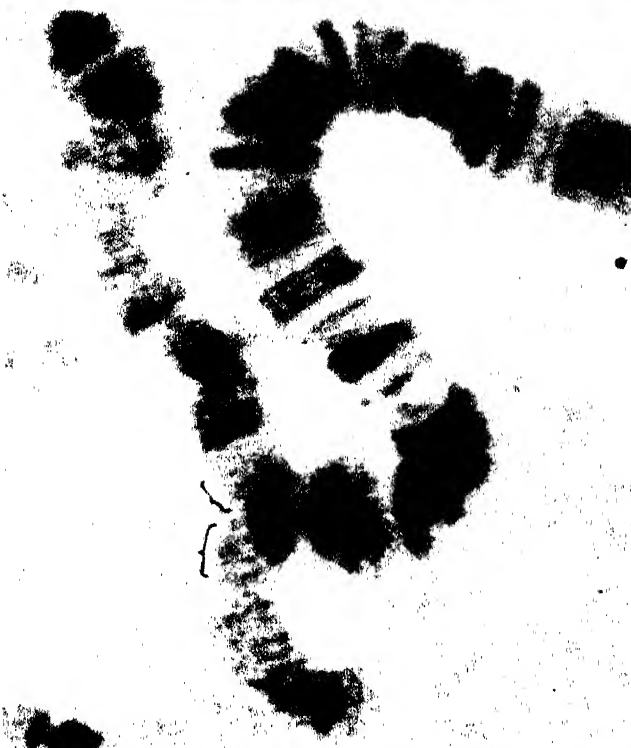
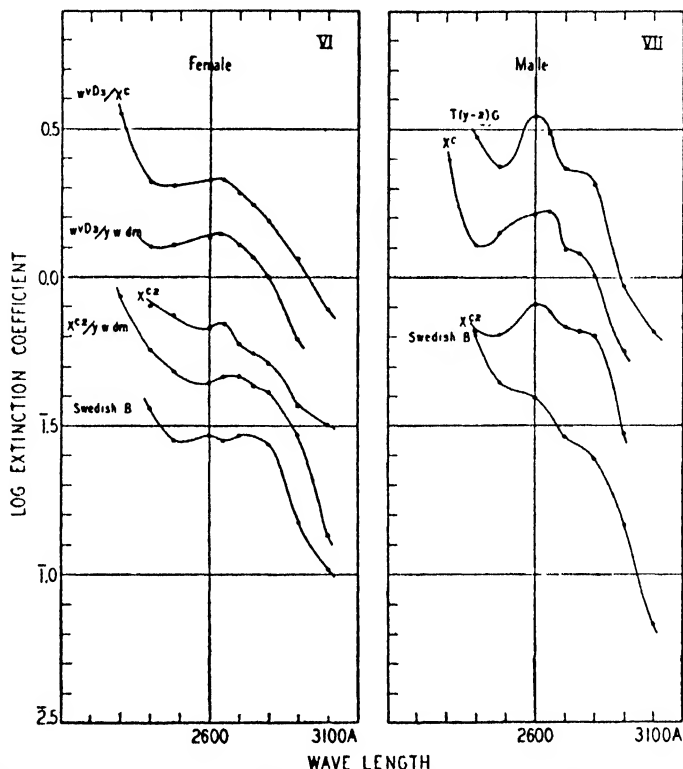


PLATE I (see facing text page for legends)

evident when it is recalled that the sources of the ribose nucleic acids for preparative work are the plant embryo, the eggs and embryos of animals, yeast, and gland cells like the pancreas. Caspersson and I have studied the relations in several cases where the comparison could be made between rapidly growing tissue and the differentiated form of the same tissue. Such, for example, are the tip of the onion root, as compared with the base; or the gut

of the oocyte itself is practically nucleic acid in nature. In this cytoplasm, the synthesis of yolk occurs and later the early rapid cleavage divisions. Now this synthesis of the cytoplasmic nucleic acids occurs in direct connection with the activity of the nucleus. It can be shown in some cases that the maximum concentration is close to the nuclear membrane. Thus in the sea urchin, the cytoplasm close to the germinal vesicle shows a much sharper nu-



TEXT FIG. 4. "Characteristic" absorption curves (logarithms of the extinction coefficients) for nucleoli in salivary gland nuclei of different genetic constituents (from Schultz, Caspersson and Aquilonius, 1940). The differences in constitution are evidenced by the shapes of the curves, showing that the proportion of nucleic acid and protein as well as the type of protein is changed in the different nucleoli.

cells of the larval *Drosophila* as compared with the rest of the imaginal disks found within. In both these cases, having little otherwise in common except the functional contrast between the two types of cell in each tissue, the rapidly dividing cells have high concentrations of nucleic acid, or better nucleo-protein, and in the mature cells, the absorption band approaches that of the proteins.

The converse case is found in such a process as oogenesis, in which the product of differentiation is a cytoplasm in which many divisions are to occur. Indeed, during cleavage in the sea urchin Brachet (1937) has shown that as the pentose nucleic acids decrease in amount, the amount of the thymonucleic acid increases. Actually in the oogenesis of such an organism as *Drosophila*, the nurse cells of the egg act almost as gland cells, whose function is the synthesis of nucleic acid, and the absorption spectrum

clic acid maximum than that farther out. And in the *Drosophila* nurse cell, the concentration around the membrane is equally sharp. In fact, we return to the evidence cited years ago for the "chromidial" hypothesis, and find the great number of cases in which basophilic material is assembled around the nucleus, which are almost certainly ribose nucleoproteins.

It is hence to be taken, even from so cursory a glance, that the distribution of the ribose nucleoproteins in cellular cytoplasm parallels the synthetic activities proceeding there. Brachet (1940b) has recently provided evidence that the case is similar in the "organizer" regions of the amphibian egg, which provides a link between the nucleoproteins and the processes of differentiation. And Caspersson, with Landström, Brandt, and Nyström (see Caspersson 1941) has shown how, correlative with pro-

tein synthesis in a variety of glandular and growing cells, the ribose nucleic acid absorption occurs. In other words, the question of the relation of the pattern of synthesis to the genes can quite precisely be stated in terms of the ribose-nucleo-proteins.

Evidence that these are genetically controlled comes from two types of experiments, one concerning the nucleic acid content of the egg cytoplasm, the other concerning the composition of the salivary gland nucleolus. It has been shown that the presence of a supernumerary Y in a female increases the nucleic acid content of her oocytes. This was done by measuring absorption at 2573 Å in groups of oocytes from different females. In ten series measured, the XXV's were consistently higher than the XX's.

The nucleolus focusses the question sharply. It turns out that there are ribose nucleic acids present in the nucleoli, linked to proteins chiefly histone in composition. In nucleoli from different rearrangements in the heterochromatic regions in *Drosophila melanogaster*, as well as in the different sexes, it was found that consistent differences existed in the absorption spectra, showing changes in the proportion of nucleic acid and in the type of protein. Now the nucleolus itself as a nuclear organelle has long been associated with synthetic processes. In fact Caspersson (1941) has recently developed the hypothesis that the process of protein synthesis proceeds by way of the histones in the nucleolus, which diffuse through the nuclear membrane to form in the cytoplasm ribose nucleic acids which are concerned with the synthesis of cytoplasmic proteins. In this way the special regions concerned with forming the nucleolus are the major producers of histones, and it is this production of histones that Caspersson believes is the function of the heterochromatic regions.

The question hinges on the presence of ribonucleic acids in the chromosomes, and as we have seen earlier the results from enzymatic digestion are not as yet conclusive. Certainly as Brachet has pointed out, from his experiments there would seem to be a reciprocal relation between the two types of nucleic acids, and it seems not unlikely that the unit of synthesis is a nucleo-protein. Were this unit of synthesis a ribose nucleoprotein its distribution in the chromosome might be at the interfaces, and the attack by ribonuclease on chromosomal structure would be expected. The special function of the desoxyribose nucleic acids might then be, not as has previously been suggested, in the formation of a chain structure by their own polymerization, but in the actual synthesis of the fibrous proteins of the chromosomes; a point of view that would agree well with the X-ray analysis carried out by Astbury and Bell. Thus the Janus molecule that is the gene, depending upon the materials available, turns its synthesis on one face to the increase of the nucleic acid component, as at the prophase of mitosis; or

conversely during the interphases, the protein component is synthesized. Thus we maintain as our unit of synthesis a nucleoprotein.

The problem of the specificities of these units has already found its pattern in the work of Warburg (1938) and others on the nucleo-protein enzymes of cellular respiration. In these cases, there are a group of different nucleotides linked to various proteins, and the experiment has been performed of putting the same nucleotide on different proteins and changing the specificity of the enzyme. The active group is the conjugated protein; neither component without the other is effective. Warburg has emphasized the special role of the proteins in determining what substrate is attacked by these enzymes. Analogously, the pattern of the amino-acids may, as Edlbacher (1938) has suggested, determine the type of new substances synthesized; but for the accomplishment of synthesis the nucleoprotein is necessary. And from the work of Ostern and his collaborators (1938) showing that the yeast nucleic acids may serve as the precursors for the coenzyme muscle adenylic acid, we have the suggestion that the different specific nucleotides of the respiratory enzymes may have their source in the nucleic acids.

The striking type of synthesis performed by the genes according to theory is of course their self-reproduction. While for the early workers on the chemistry of the nucleus this was a unique fact, our position is more fortunate. The workers on the viruses and on the bacteriophage have shown these to be nucleoprotein in nature; and Menke's (1939) analysis of chloroplasts is to be interpreted as showing that these self-reproducing units also contain nucleoprotein. It is to be noted that the nucleic acids concerned are the ribose nucleic acids, and that the protein is not histone in nature but more complex: evidence supporting the view that the role of the desoxyribose nucleic acids is to be sought in the synthesis of the fibrous proteins peculiar to the chromosomes. In any event, the parallel between the occurrence of nucleoprotein and genic properties holds. We have a chain macromolecule, as Koltzoff (1928) long ago suggested, and Wrinch and others have elaborated. The specificities of the genes reside in the nucleoprotein portion, and the continuous structure is a protein fiber.

One of the most curious properties of this chain of connected specificities is the apparent adjustment of the rates of self-reproduction. This is of course completely a deduction from the fact that the two daughter chromosomes at mitosis are apparently equal. Whether at all mitoses the divisions are equal is only inferred. The problem has a special point in the cases of endomitosis, of which in general it would seem by now most differentiated tissues may offer examples, correlated with their activity: the more active the cell in synthesis the higher the degree of endomitosis. Now it is possible to pose the question whether in the endomitotic cycle, all genes

divide at the same rate, and I have recently found evidence that in fact in the nurse cell endomitosis in *Drosophila melanogaster*, described by Painter and Reindorp (1939), the heterochromatic regions divide more slowly than the others. The simplest proof comes from the examination of females containing different numbers of Y chromosomes. In the endo-interphases of XX females, the individual heterochromatic regions may be seen as heterochromomere like bodies, with approximately four to eight strands projecting from them in the nuclei that according to Painter and Reindorp have 512 representatives of each chromosome. That this is actually due to a slower rate of division on the part of the heterochromatin is reaffirmed by study of the different stages of endomitosis upon which I will not enter here. In the XXY female with the additional heterochromatic chromosome the Y is seen as a double and in the largest nuclei perhaps octuple series of connected two-armed bodies. In the XXYY female there are double this number. The evidence seems good therefore that the Y, and the other main heterochromatic regions of *D. melanogaster* do not divide at the same rate as the rest of the chromosome in these cells. It is conceivable that this is a function of their relation to the centromere, and a result of the failure of a stimulus from the centrosome for separation of the centromeres. At any rate, it is significant that so far as the evidence goes (more study may be necessary), the heterochromatin of the salivary gland chromosomes does not behave in this way. It appears that the internal reproduction of different types of genes may be different in the different tissues.

It is suggestive that the "heterochromatic" regions which have the slow reproductive rate in the nurse cells, are also distinguished by the relatively high amount of nucleic acid that they synthesize. The hypothesis has been made, on the basis of their effects on cytoplasmic and nucleolar nucleic acids, that their main function is concerned with the nucleic acid metabolism of the cell. It may be, and we shall return to this point, that protein production and nucleic acid production at a given locus in the chromosome are inversely correlated and that the rate of gene reproduction for a locus has as one limiting factor the amount of nucleic acid required to be synthesized for reproduction at that locus.

The discussion thus far has tacitly assumed that the chromosomes synthesize their own nucleic acid. This assumption is based on evidence from two sources: one (Caspersson, 1939) the meiotic pro-phases in the grasshopper which show an increase of nucleic acid content during the earliest pro-phases. The other evidence is from the growth of the salivary gland cells, showing that there is more nucleic acid in the nucleus alone of the mature cell than in all the young cell (Caspersson and Schultz). As the nucleic acid of the nucleus is higher in concentration than that of the cytoplasm, it seems most

likely that the synthesis of nucleic acid which has taken place is a local synthesis in the nucleus, and not a transport from the cytoplasm. The argument is reinforced by the fact of the concentration gradient of cytoplasmic nucleic acids around the nuclear membrane, which is in accord with the view that the nucleus is the synthetic center. From this point of view it is all the more evident that static theories of chromosome structure, based upon one stage of mitosis, are not very helpful in the evaluation of the relation of the substances in the nucleus to the genes.

THE CORRELATION OF GENETIC CHANGES WITH THE NUCLEOPROTEIN METABOLISM OF THE CHROMOSOMES

But the evidence from general correlations of the type we have been considering leaves much to be desired. It can be extended further: for example, the relation of the mutation process to the reversible stage of a protein denaturation process might be considered. It will be evident from a consideration of the data, that, broadly speaking, denaturing agents either kill the organisms or cause mutations; and that the evidence so far from the ultraviolet mutation work like that on the denaturation of conjugated proteins favors the especial importance of the nucleic acid prosthetic group in producing stable changes in the pattern of synthesis, that is, mutations—a relation similar to that encountered in the denaturation of conjugated proteins. It would however be most desirable if there were a system in which changes in the behavior of specific genes could be correlated with the nucleoprotein metabolism of the chromosomes. And there is such material in certain of the mosaic, or variegated, races of *Drosophila*; and in my belief, although the question is disputed, the evidence is good for a correlation between the change in the genes and the change in the nucleoproteins.

It would be too long a task to review in detail the evidence on the subject, which will in any case be under further discussion at this symposium. Briefly, the case is the following. A whole group of translocations as variegated types are all but a very few found to be associated with breakages around the spindle attachments, in the regions which we have already discussed as heterochromatic (Schultz, 1934, 1936, and others). Now these heterochromatic regions have been shown to have a definite relation to the nucleic acid metabolism of the cell. And it is not surprising in retrospect, although it was one of the earlier bits of evidence for the idea, that the bands in the salivary gland chromosomes should show correlated changes in their behavior. This is apparent in stained preparations and there has been confirmation of some of the results by Prokofyeva (1939). The measurements which Caspersson and I have made on a case selected for its convenience showed that at higher temperatures, where the spotting is a less extreme departure from the wild type,

the nucleic acid content of the bands was increased; that under more extreme conditions there might be an apparent loss. It is my feeling that we have here still the beginning of an attack: for the question of the interrelations between the affected bands and their heterochromatic neighbors has scarcely begun to be analyzed, and it is obvious that many complexities await us. But when the data are correlated with the information concerning the slow rate of division of the heterochromatic regions in the nurse cells of the ovary, the relation of the darkening of the bands to gene reproduction previously surmised now becomes more evident. Apparently a change in rate or mode of reproduction, manifest in the changed nucleic acid balance, is at the root of the changes which lead to the mosaic spots. On this basis the effect of an increase in the amount of cytoplasmic nucleic acids, due to an extra Y in the mother, could be to increase the rate of reproduction in the affected regions, and restore the normal situation. This is in fact what happens in the presence of an extra Y.

We have then traced a parallel between the properties of the nucleoproteins and those of the genes, and discussed briefly the beginnings of the detailed analysis of these interrelations. Indeed it is possible to go farther, and see in the synthesis of a nuclear membrane at the telophase of each mitotic cycle the establishment of a specific mosaic for synthesis related to the protein sheath of the nucleoprotein in the chromosome. Caspersson has recently interpreted the absorption data of interphase and metaphase chromosomes as consistent with the old evidence of Miescher and Kossel on spermatogenesis. What happens in that case is a transformation of the protein content of the testis from the more complex to the simpler types found in the chromosomes. Caspersson (1941) suggests that this occurs during every mitosis, and that contrariwise, at telophase, there is a new synthesis of complex proteins by the nucleoproteins of the chromosomes. This is of course stated in chemical terms, the conclusion which was arrived at from a consideration of the relation between the bands and the interband spaces in the variegational translocations. In view of the results from the digestion experiments with ribonuclease, the data must, however, be restated in terms of the synthesis of a protein sheath related to the nucleolar material, and here the analysis has not yet proceeded far enough to permit conclusion. The basic points remain however. At the present time, the properties of the genes and of nucleoprotein metabolism are evidently parallel: specificity, self-reproduction, relations to synthesis, and distribution of the nucleoproteins in the cell, all are what they should be were the genes nucleoproteins. It would seem therefore that our present task is to develop the physiology of the nucleoproteins into an effective physiology of the genes.

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DISCUSSION

GLASS: In the last table (total extinction in halves of bands), are the determinations made from a single preparation?

SCHULTZ: Yes.

GLASS: We know little about the extent of random variation in such preparations; much variability usually appears. Have you a large number of readings on different preparations?

SCHULTZ: I have made statistical studies where the staining of the translocated and normal chro-

mosomes could be compared within the same cell, and found these differences consistent in the stained material. Furthermore Prokofyeva has seen the same sort of thing. The problem of random variation is then taken care of by this previous work.

MULLER: The nearer you get to the heterochromatin, the more the phenotypic spotting is affected, but it is hard to see the relation between this and the degree of effect on the chromosome banding. For according to your observations the transfer of a band to the vicinity of a heterochromatic region results in its becoming darker, but its still closer proximity to a heterochromatic region results in its becoming lighter again. This seems like blowing both hot and cold. How then can you say what your theoretical expectation is?

SCHULTZ: In the cases where mottling is slight, darkening of the bands appears; where mottling is extreme and approaches the effect of deficiency for the locus, the absorption decreases. This is consistent with the expectation that a band becoming heterochromatic, and therefore slowed down in its reproductive rate, will first increase in nucleic acid content like the heterochromatic regions; in more extreme cases it will be slowed down in reproductive rate sufficiently to give less total absorption in the mature chromosome.

(Rest of discussion deferred until after the paper of Cole and Sutton).

THE ABSORPTION OF ULTRAVIOLET RADIATION BY BANDS OF THE SALIVARY GLAND CHROMO- SOMES OF *DROSOPHILA MELANOGASTER*

PETER A. COLE AND EILEEN SUTTON

In *Drosophila melanogaster* a type of variegation is found which is associated with known autonomous genes and in which the tissues affected by these genes show a mixture of wild-type and mutant cells. Studies by Demerec (1939) indicate that the normal action of the wild-type allele concerned is partially or completely suppressed in the early development of the tissue, but may be restored locally at later divisions. Thus, patches of wild-type tissue, one or more cells in extent, occur on a background of cells of the mutant type.

The ratio of wild-type to mutant cells varies in different stocks, and in a given stock the rate of reversion to wild-type is proportional to the temperature at which the flies are raised, high temperature causing a greater, and low temperature a lesser amount of wild-type tissue.

Schultz (1936) was responsible for showing the correlation of this kind of variegation with chromosome rearrangements due to breaks, on the one hand, close to the affected loci, and on the other hand, in the heterochromatic regions of the chromosomes. The mottling is caused in some way by the presence of heterochromatin near to the affected locus, when a recessive allele for that locus is present in the normal chromosome.

The purpose of the work described here was to study the salivary gland chromosomes in a stock showing mottling of this type with a view to detecting any change in bands translocated from their normal position to heterochromatic and euchromatic regions. It had already been suggested by Schultz (1938) that these bands tend to increase their nucleic acid content and in extreme cases become "heterochromatinized" (see also Prokofyeva-Belgovskaya, 1937). This phenomenon was supposed to be responsible for the instability of the genes represented by these bands. A preliminary study by the junior author (Sutton, 1940) by means of the visible light microscope, failed to detect any significant changes in translocated bands of some mottled stocks at 18° C. This paper presents some evidence on the nucleic acid content of the bands, obtained by the use of the ultraviolet microscope. Further and more critical experiments are being planned.

MATERIALS AND METHODS

For this study we used a stock, white-mottled 258-21, which shows mottling for the X-chromosome loci, *w*, *rst*, *fa* and *dm*.

The females of this stock are heterozygous for an X-ray induced chromosome rearrangement, in which the X-chromosome was broken between 3E5 and 6, and the fourth chromosome was broken at the chromocenter. The tip of the X-chromosome with the loci *w*, *rst*, *fa* and *dm* was reattached to the chromocenter of the fourth, while the distal part of the fourth was attached to the base of the X-chromosome preceding 3E6. The second X-chromosome in the female is cytologically normal.

At room temperature the eyes of females of this stock heterozygous for *w* show white and cherry facets characteristic of the mutant alleles *w* and *w^{ch}* at this locus, with large patches of wild-type tissue. At the temperature at which the larvae were raised for this experiment (about 18° C) the proportion of mutant tissue is increased and as much as one-quarter of the eye may be white, the majority of the facets being cherry colored. With genes nearer to the break, as is the case for *fa* and *dm*, a greater amount of mutant tissue is present than in the case of the *w* locus. The material therefore seemed favorable for observing any effect on the chromosome bands, associated with the suppression of gene activity in the neighborhood of the break.

As a control, the stock Notch 264-69 was used. In this stock females were heterozygous for a translocation with a break in X between 3C7 and 8 (about 10 bands to the left of the breakage point in white-mottled 258-21) and the second break in the right arm of the second chromosome, between 44C4 and 5. In this case the *w* and *fa* loci were translocated to euchromatin of 2R and no mottling effect was obtained. The *w⁺* gene was apparently unchanged, while the *fa* locus showed a stable change from wild-type to the recessive allele.

As all salivary gland preparations were made from female larvae heterozygous for the rearrangements, the translocated bands could be compared with the corresponding bands in the normal X-chromosome.

The glands were fixed in 45 percent acetic acid for

PLATE I—Figs. 1-5

(1) Microphotographic apparatus; (2) Part of salivary gland chromosome photographed at different wavelengths; (3) Tip of X chromosome translocated to chromocenter, showing dotted band 3C9·10 (lower arrow) partially obscured by heterochromatin (top arrow); (4) Heterochromatic translocation *w*-mottled 258-21. Arrows show bands measured for comparison of 3E1 in normal and translocated chromosome and for variation within the band in 3A1·2 and 3A4 (x 2000); (5) Euchromatic translocation N264-69. Arrows show bands of 3A and 3 C measured for comparison in normal and translocated chromosome, and bands in normal 3L differing visibly in density (x 3000).

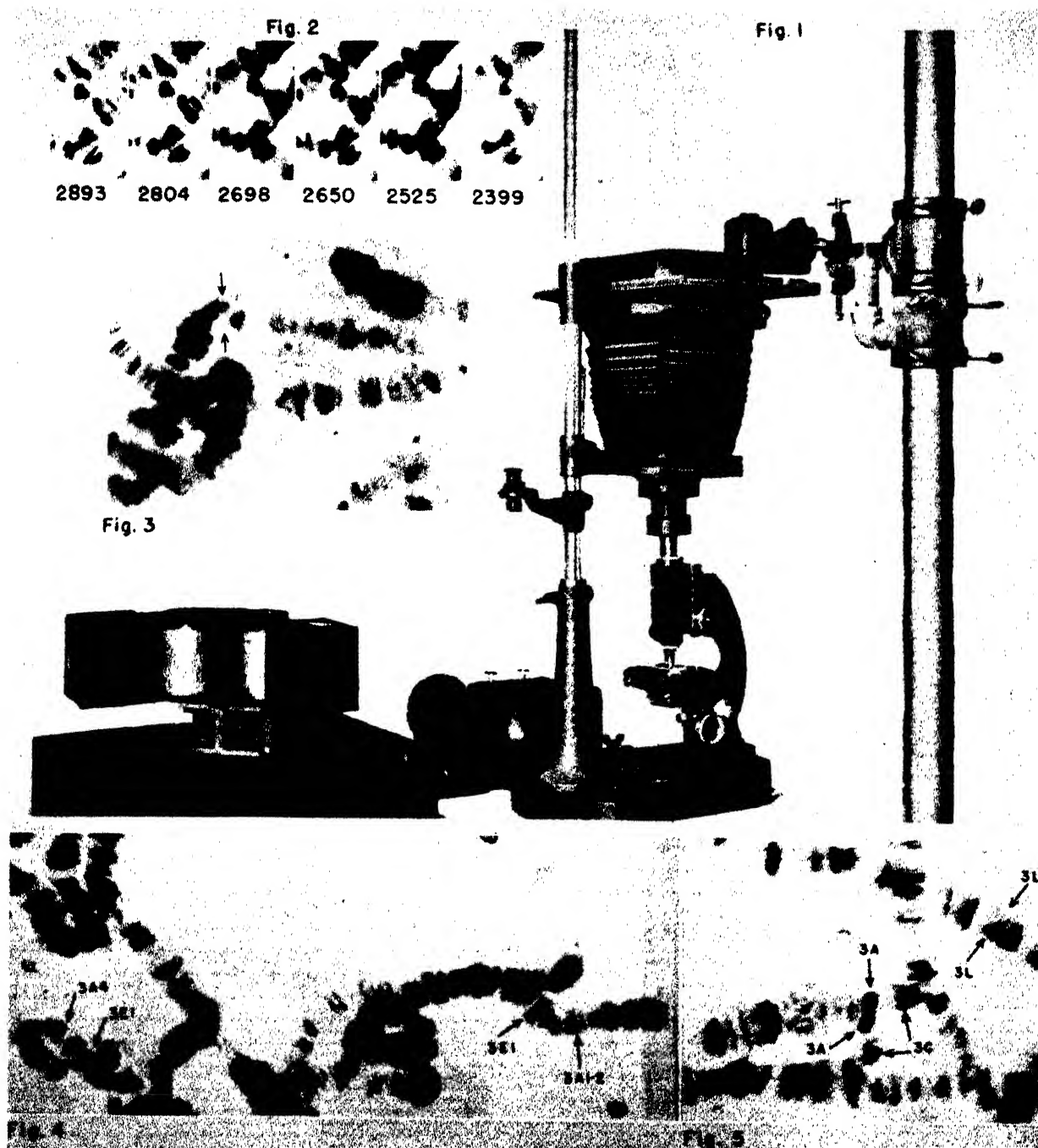
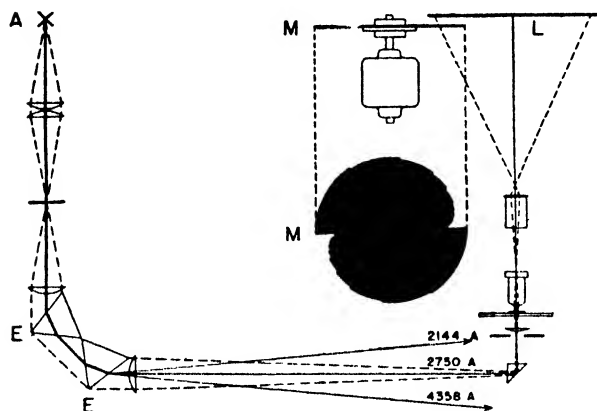


PLATE I—Figs. 1-5 (Legends on page 66)

about 10 minutes, and smeared in the usual way on quartz slides under quartz coverslips.

Photographs of the smears were made using a Zeiss ultraviolet microscope (Plate I, fig. 1) with a 5X eyepiece and a 1.7 mm objective of 1.25 N.a. or a 2.5 mm objective of 0.85 N.a. The bellows extension of the camera was 30 cm giving a 900X



TEXT-FIG. 1. Diagram of microphotographic apparatus. A, source of light; E, monochromator; L, photographic plate; M, step sector disc.

or 600X magnification at 2652 Å with the 1.7 mm and 2.5 mm objectives respectively. The actual resolving power with these preparations at 2652 Å is between 0.1 and 0.2 microns. An H-3 mercury arc with outer glass jacket removed and a cadmium spark in air served as sources of radiation. Each wavelength desired was isolated by means of a large crystal quartz monochromator made available to the authors by the Washington Biophysical Institute.

Photographs were taken at from six to fourteen wavelengths between 2967 and 2199 Å, refocussing at each wavelength to 1/2 micron. Since the material was fixed, no trouble was encountered due to alteration of material by ultraviolet radiation.

A focal plane step sector disk was used for plate calibration (Webb, 1933; Cole and Brackett, 1940). Densitometry of the photographs was carried out with a modified "spotting" Bausch and Lomb densitometer. A circular area equivalent to 0.4 microns in diameter in the object plane of the microscope was used to determine the density of any point. Only areas near the centers of the bands were measured to eliminate as far as possible diffraction and refraction effects. A complete treatment of this point is given by Caspersson (1936, 1940). For a description of the technique employed in the operation of the microscope, see Cole and Brackett (1940).

RESULTS

Several nuclei from each stock were photographed over a series of wavelengths between 2199 and

2967 Å. A set of photographs from one figure is shown in the plate (fig. 2). From the series of calibrated plates of a given figure, absorption curves for certain bands were determined. Text-figure 2a shows some of these curves, compared with a curve for yeast nucleic acid obtained from Dr. Greenstein (fig. 2b).

Special attention was paid to the absorption at 2650 Å of bands adjacent to the break in the X-chromosome. A set of measurements of such bands was made from six nuclei of the white-mottled stock, 258-21. When bands are translocated to heterochromatin they are more difficult to distinguish, because they may be covered by distortion of the heterochromatin when the glands are smeared (fig. 3). Further, Schultz has shown a loss of distinctness of the bands nearest to heterochromatin in this stock. Light bands such as those in 3E are extremely difficult to detect, and it was not possible to make comparative measurements of these bands in any of the figures. Measurements were made of 3E1·2, which was the nearest heavy band to the left of the break. As the loci of *w*, *fa* and *dm*, still further from the break, showed mottling in this stock, and as the effect is greatest near to the break, any effect on the bands should be apparent in 3E1·2 (fig. 4).

A second set of measurements was made from nuclei from the control stock, Notch 264-69. Here the break was immediately to the right of band 3C7, which was reattached to 2R. Measurements were made of bands to the left of the break in both the normal and translocated position (fig. 5).

It was observed from the plates that there were occasionally marked differences in the density of two corresponding bands in the two synapsed strands of a normal chromosome (fig. 5). A set of measurements was obtained for these differences, which, being clearly visible, would supposedly show the extreme limits of random variation in normal bands.

It was also found that a band in a single unsynapsed chromosome might vary in density across half of its width (fig. 4). This occurred both in normal and in translocated chromosomes, and was obviously independent of a heterochromatin effect. The phenomenon is interesting, because it might be interpreted as showing an initial two-strand condition of the salivary gland chromosome. A fourth group of measurements was made of such cases.

Table 1 shows these four sets of measurements. In each set the densities of the two corresponding bands, or parts of bands, are given in columns 2b and 3b, with the difference between these two values in column 4b. The consistency of the measurements was checked by comparing these data at 2650 Å with the corresponding data obtained by a shift of wavelength to 2570 Å. The densities and differences at 2570 Å are shown in columns 2a, 3a and 4a. In only one case (Group 1, 3rd line) is there a marked change in the difference obtained. In general, the differences at the two wavelengths are nearly the

same, and the results of averaging the densities at these two points, on either side of the maximum of the absorption curve, are substantially the same as those obtained from the 2650 Å values alone.

It will be seen from the table that the differences

The variation in this group is also of about the same order as that in the group of visible differences in bands of normal chromosomes (Group 3). The bands of the heterochromatic translocation do not show greater differences in density than the normal

TABLE 1. MEASUREMENTS OF DENSITY OF ABSORPTION IN BANDS OF SALIVARY GLAND CHROMOSOMES AT 2650 Å AND 2570 Å

Group 1. Heterochromatic translocation w-mottled 258-21						
Band	Density of norm. chr.		Density of transloc. chr.		Difference: norm-transloc.	
	(a) 2570 Å	(b) 2650 Å	(a) 2570 Å	(b) 2650 Å	(a) 2570 Å	(b) 2650 Å
3C2·3	0.43	0.47	0.41	0.43	+0.02	+0.04
3E1·2	0.45	0.47	0.44	0.46	+0.01	+0.01
3E1·2	0.56	0.47	0.50	0.50	+0.06	-0.03
3E1·2	0.29	0.27	0.42	0.46	-0.13	-0.19
3E1·2	—	0.40	—	0.39	—	+0.01
3E1·2	—	0.27	—	0.20	—	+0.07
Average		0.392		0.407		-0.015
Group 2. Euchromatic translocation N264-69						
Band	Density of norm. chr.		Density of transloc. chr.		Difference: norm-transloc.	
	(a) 2570 Å	(b) 2650 Å	(a) 2570 Å	(b) 2650 Å	(a) 2570 Å	(b) 2650 Å
3A4	0.42	0.42	0.32	0.30	+0.10	+0.12
3C2·3	0.38	0.42	0.66	0.68	-0.28	-0.26
3C5·6	0.445	0.42	0.400	0.39	+0.045	+0.03
3A3	0.39	0.45	0.41	0.50	-0.02	-0.05
3C2·3	0.43	0.48	0.42	0.45	+0.01	+0.03
Average		0.438		0.464		-0.026
Group 3. Normal chromosomes: visible differences						
Band	Density of bands				Difference	
	(1a) 2570 Å	(1b) 2650 Å	(2a) 2570 Å	(2b) 2650 Å	(a) 2570 Å	(b) 2650 Å
21C1·2	0.26	0.35	0.27	0.28	0.01	0.07
21E1·2	0.30	0.34	0.26	0.30	0.04	0.04
3L	0.44	0.45	0.24	0.26	0.20	0.19
59E1·2	0.45	0.46	0.33	0.31	0.12	0.14
68C1·2	0.43	0.42	0.31	0.29	0.12	0.13
15D1·2	0.49	0.49	0.43	0.43	0.06	0.06
15F1·2	0.45	0.45	0.41	0.41	0.04	0.04
Average		0.423		0.326		0.097
Group 4. Unsynapsed chromosomes: visible differences within bands						
Band	Density				Difference	
	(1a) 2570 Å	(1b) 2650 Å	(2a) 2570 Å	(2b) 2650 Å	(a) 2570 Å	(b) 2650 Å
norm. chr. 3A1·2	0.34	0.325	0.20	0.200	0.14	0.125
" " 3D5·6	0.30	0.32	0.21	0.20	0.09	0.12
" " 3A1·2	0.53	0.53	0.28	0.28	0.25	0.25
transloc. chr. 3A1·2	0.49	0.49	0.33	0.37	0.16	0.12
Average		0.416		0.262		0.154

in the case of the heterochromatic translocation are of the same order of magnitude as those in the control material of the euchromatic translocation. The bands adjacent to heterochromatin are sometimes denser and sometimes less dense than those in the normal chromosome, and there is no apparent tendency for the translocated bands to vary in one direction more than the other.

bands. The averages for the group do not indicate any significant difference between groups.

The picture we have is of random variation in the bands, whether they are in a normal position or rearranged. This is borne out by the variation within a single band of an unsynapsed chromosome (Group 4), this variation too being of the same order as that found in the other groups.

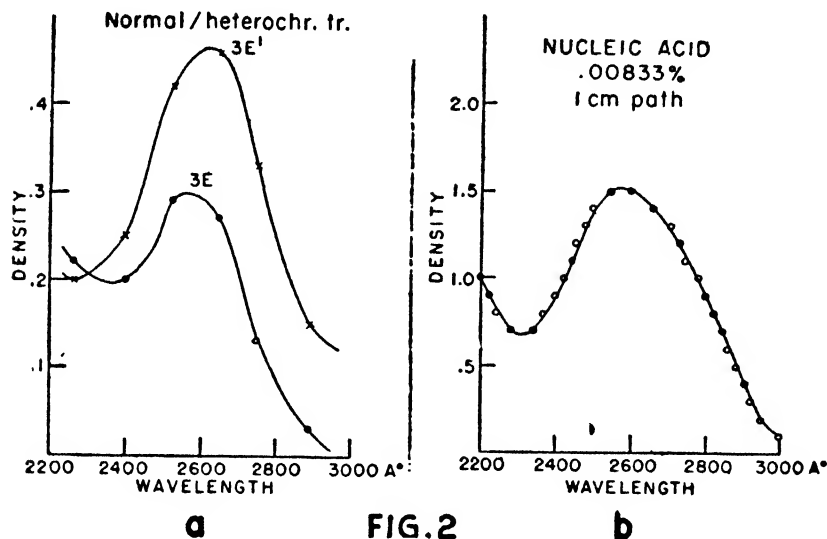


FIG. 2

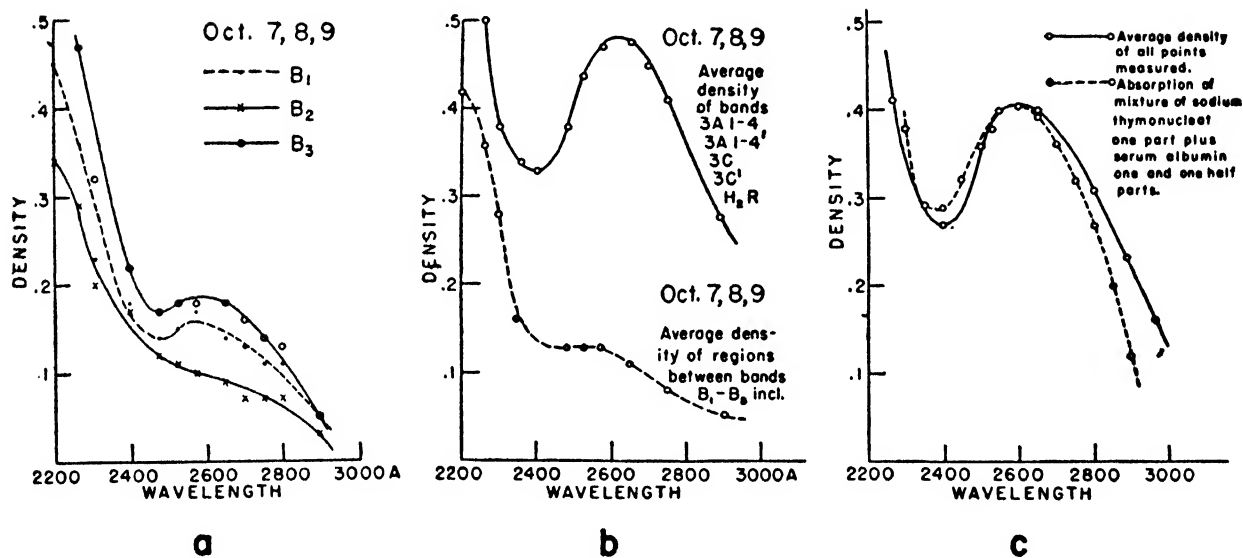


FIG. 3

TEXT-FIG. 2. (a) Absorption curves of bands of the salivary gland chromosomes; (b) Absorption curve of yeast nucleic acid.

TEXT-FIG. 3. (a) Absorption curves of interband spaces; (b) Comparison of average absorption curves for bands (5 measurements) and interband spaces (5 measurements) from the same plate; (c) Comparison of average absorption curve for bands with the curve obtained from a synthetic protein—thymonucleate mixture.

If the heterochromatin has any effect on the nucleic acid content of neighboring bands, this effect is not detectable because of the random variation, which is very evident.

Several of the conditions governing this experiment may have served to emphasize this random variation at the expense of any consistent differences in density.

In the first place, the method of measuring a restricted portion of each band, rather than the whole, tends to reduce the value of comparisons, because, owing to the variation within bands, the area measured may not be representative of the

band as a whole. This method was adopted, however, in order to avoid errors due to refraction and diffraction.

A second limiting factor is the difficulty of measuring bands near to the chromocenter. This difficulty arises from two sources—first, the frequent superposition of one chromosome on another in the region of the chromocenter; second, and more important, the specific phenomenon of indistinct banding described by Schultz. This phenomenon occurs in our material, but not to an extreme degree, and in many figures all bands up to 3E1.2 retain their characteristic euchromatic appearance.

It may be concluded that more critical evidence can be obtained by using the same mottled stock under different conditions which decrease or increase the amount of mutant tissue (high and low temperature, presence and absence of extra Y chromosomes or genetic modifiers), thus making comparisons between stocks showing known degrees of mottling.

It has been shown by Mazia and Jaeger (1939) and Caspersson (1940) that the salivary gland chromosomes have a skeleton of protein material which extends throughout their length. Only part of the absorption by the bands at 2650 Å is due to nucleic acid, therefore, and part is due to their protein backbone. By measuring the absorption of the spaces between bands it is possible to estimate how much of the absorption in the bands is due to this backbone constituent. Text-figure 3a shows curves for a number of points between the bands, over a series of wavelengths.

The maximum at 2650 Å suggests that some nucleic acid may be present between the bands. On the other hand, this may be due to the inclusion of very light bands in the places measured. In any case, the nucleic acid content is low, and there is possibly five to ten times as much protein present as nucleic acid.

In any particular measurement at 2650 Å, either between or within the bands, the density of the protein skeleton probably amounts to about 0.1. The rest of the absorption in the bands may be due to nucleic acid. In that case, if we plot an average absorption curve for the bands, and an average for the spaces between bands, we might expect that the difference between these curves would give a curve closely resembling that of nucleic acid. The curves do not correspond very well, however, as is shown by a comparison of Text-figures 2b and 3b. A possible explanation for this discrepancy is that there is a concentration of protein, as well as of nucleic acid, in the bands. It is of interest that Caspersson's work shows variation in the type of protein found in different regions of the chromosome.

A further comparison was made by preparing a curve from the averages of all points measured at each different wavelength. This curve was compared with that obtained from a mixture of two parts sodium thymonucleate and three parts serum albumin (fig. 3c). The choice of a protein thymonucleate mixture was somewhat arbitrary. It is merely a mixture which gives a curve, corresponding fairly closely with that obtained from the chromosome measurements. Other combinations were tried, and found to give a less satisfactory fit.

It may be estimated from these results that the nucleic acid content of the bands is perhaps 70 per cent of the protein content.

SUMMARY

The results show that all bands, whatever their position, vary considerably in their nucleic acid

content. The nucleic acid content of the bands is high and they may also have a higher concentration of proteins than the rest of the chromosome.

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DISCUSSION

SCHULTZ: In these experiments selection has been made of figures where the piece translocated into the heterochromatin can be seen, therefore a comparison cannot be made of two sets of results. This is particularly true of the 3E1 band, where the cases were selected in which a visible reaction with heterochromatin had not occurred.

A euchromatic translocation is not a valid control. One should use as a control the same translocation under conditions where variegation is at a minimum.

Differences between densities of bands may be due to differences within the translocation itself, where the differences are great, and also due to the fact that in the last division of the salivary chromosomes, some increase in differences might occur. I should like to point out that measurements of a single point in each band cannot be used as an index of total quantity of material in the band.

DEMEREC: The 3E1-2 band is very dark, and there is no difficulty in distinguishing it.

SUTTON: There was no selection in this material. We were able to use every figure with the exception of those in which another chromosome was overlying the bands.

SCHULTZ: I did not mean that there has been a conscious selection of figures of a given type, but that selection would have had to be made of figures where the band could be seen, in order to make these measurements. The reason for bringing this up is that heterochromatic regions have a characteristic network structure. In translocations to the heterochromatin, one gets a turnover of the type of bands to that characteristic of heterochromatin, instead of clear bands. Sutton's overlying heterochromatin may actually be euchromatin so altered. The difference between the two possibilities should be easy to see. In our material, the conditions governing the selection of figures were twisting of

chromosomes, etc., not an effect of the heterochromatin, in other words, all cases that could be measured were used.

DEMEREK: How many figures did Caspersson and Schultz measure?

SCHULTZ: Detailed measurements were made of eight figures.

STERN: (To Sutton) How did you select normal chromosomes for controls?

SUTTON: We chose places on the normal chromosome where there were visible differences, in order to study the extreme of variability.

MULLER: I would like to emphasize the fact that there is more to be studied than merely the concentration of nucleic acid. In studies made in Russia of these eversporting displacements, we came to the same general conclusion as Schultz (Muller, 1935, *PROC. INTERN. CONGRESS PHYSIOL.*) that translocation to a position near the chromocenter gave rise to variegation. The translocated region partook of

the nature of heterochromatin in various respects, acquiring the latter's granular appearance, and its other distinctive characteristics, to a degree depending upon how near it was to the heterochromatin. It is hazardous as yet to ascribe the cause of variegation to any one of the various known properties of heterochromatic regions, such as the difference in amount of nucleic acid in the bands.

KAUFMANN: In our X-ray induced translocations, we occasionally see the phenomenon referred to by Schultz and Muller. Sutton has analyzed figures obtained in one rearrangement, and finds that some of the figures show bands comparable in intensity to those in normal positions, while the other half become more densely stained.

SUTTON: In Kaufmann's case, the affected bands may themselves be heterochromatic material since they are in the bulb region near the tip of the X. In another group of material of heterochromatic translocations, this effect is not shown at all.

SPONTANEOUS ALTERATIONS IN CHROMOSOME SIZE AND FORM IN ZEA MAYS

BARBARA McCLINTOCK

Spontaneous aberrations in maize leading to changes in size and form of the chromosomes have not been investigated from the point of view of determining, systematically, the frequency and positions of breakages and reunions of broken ends of the chromosomes of the complement, as has been done in *Tradescantia* (Giles, 1940), in *Allium* (Nichols, 1941) and in other forms (Darlington and Upcott, 1941). Nevertheless, through studies of various problems not directed toward this goal, much has been learned of the process underlying the origin of changes in size and form of the chromosomes of maize which are not conditioned by the usual methods of inducing aberrations, such as X-radiation, ultraviolet radiation, high temperatures and aging.

In the early cytological studies of maize, it became clear that spontaneous aberrations were occurring to give rise to various types of altered chromosomes. In many cases, the time of occurrence or the conditions which gave rise to the alteration were not known. These aberrations were first observed in various plants of particular strains which were under cytogenetic investigation. These aberrations included reciprocal translocations, inversions, deficiencies, ring-chromosomes, a duplication, fragments, and a secondary trisome. Through further studies, it became apparent that chromosome modifications were occurring in individual plants under investigation. A single plant of a culture may show one of the various types of aberrations mentioned above. Although, in some cases, it could not be determined whether all of the cells of the plant possessed the aberration, in other cases it was determined that the plant was sectorial for the modification. In these latter cases, it was obvious that the modification occurred during the development of the individual plant. The factors responsible for these spontaneous aberrations were not apparent in any of these cases. However, there are types of chromosomal aberrations which are induced by known factors or are correlated with known conditions. These will be considered under appropriate headings in the following discussion.

SPONTANEOUS CHROMOSOME ABERRATIONS UNDER GENIC CONTROL

There are two well investigated cases which indicate that the rate of spontaneous chromosome aberration in maize may be controlled by the genic composition of the nucleus. The first case is strikingly illustrated by the *sticky* gene studied by Beadle (1932, 1937). *Sticky* is a recessive mutant

located in chromosome 4 which causes a tremendous increase in the rate of spontaneous chromosome aberration in all types of tissues. At the first meiotic anaphase in homozygous plants, the chromosomes appear adhered to one another. This sticking together of the chromosomes of the complement suggested the designation *sticky* for this mutant. As a consequence of this sticking, many of the chromosomes are ruptured during the meiotic anaphase. In the mitotic divisions, numerous types of chromosomal aberrations were observed in plants homozygous for the *sticky* mutant. The continued production of spontaneous aberrations during development causes plants homozygous for *sticky* to be stunted in growth and to possess numerous streaks of tissues with altered phenotypes. The endosperm tissues are likewise a mosaic of various types of aberrant cells. The extremely high rate of spontaneous chromosome alterations, both in the sporophytic and endosperm tissues, undoubtedly is the cause of the observed phenotypic alterations of the cells and tissues, for various grades of chromosomal unbalance must be present in these cells. It is likewise of particular interest to note that the *sticky* mutant is responsible for a marked increase in the rate of spontaneous mutation.

The second case of spontaneous chromosome alteration under genic control has been studied by Jones (1937, 1940). In some of his strains of maize, the endosperm tissues give unmistakable evidence of a high rate of spontaneous chromosome aberration. Unlike the *sticky* mutant, the high rate of chromosome aberration appears to be confined to the endosperm tissues alone. The genetic evidence indicates that reciprocal translocations are occurring between non-homologous chromosomes and that chromosomes with unstable broken ends are likewise produced. Other types of chromosomal aberrations could not be detected genetically. Cytological observations of the endosperm tissues of these plants have produced direct evidence of a high rate of spontaneous chromosome aberration (Clark and Copeland, 1940).

It is needless to say that any systematic study of spontaneous chromosome aberration in maize must be considered with reference to the genic composition of the plants under investigation.

CHROMOSOME ALTERATIONS INDUCED BY CROSSING OVER BETWEEN HOMOLOGOUS SEGMENTS OF CHROMOSOMES

The normal process of crossing-over may be responsible for the production of chromosomes with

altered sizes and forms. When individual plants are heterozygous for an inversion, a duplication or some rearrangement in the linear organization of the chromosome, predictable types of chromosomal alterations may follow crossing-over between homologous segments of chromosomes. Although structural heterozygosity greatly increases the rate of production of altered chromosomes following crossing-over, it will be shown that chromosome aberrations likewise may be induced within a normal complement following the regular process of crossing-over. Several examples illustrating the part that crossing-over plays in the production of chromosomal aberrations will be considered.

more deficient than the broken chromatid entering the sister nucleus. The latter broken chromatid will possess a duplication besides a deficiency. Several such inversions have been investigated in maize (McClintock 1933, 1938b). In several cases, a deficient broken chromosome, presumably derived from crossing over within an inverted segment, has been recovered in the following plant generation.

The breakage of a chromosome at a meiotic anaphase is the starting point in the production of chromosomes with various modifications of size and chromatin constitution. The direct cause of these modifications is related to the subsequent behavior of the broken end. When a chromatid is broken at a

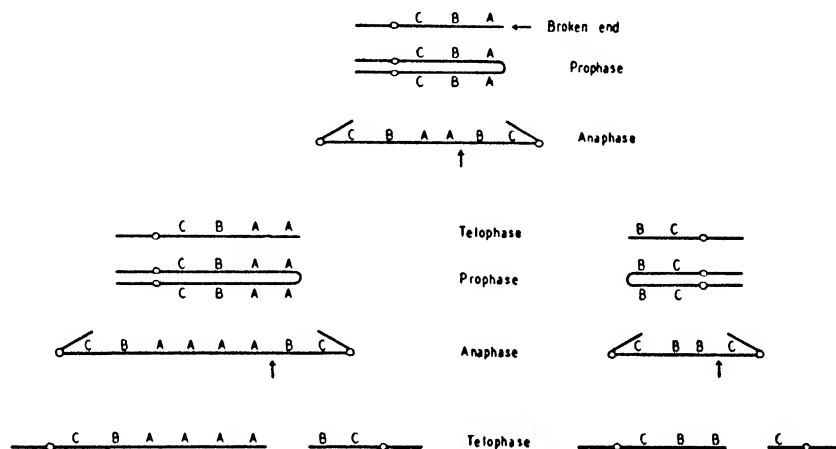


FIG. 1. Diagrammatic representation of the method by which a chromosome with a broken end gives rise to chromosomes with altered sizes and constitutions. The diagram at the top of the figure represents a chromosome with a broken end. The centromere is represented by the clear oval. The organization of the arm with the broken end is represented by A, B and C, A being adjacent to the broken end. Following reduplication of the chromosome, the two sister chromatids are fused at the position of previous breakage (Prophase, second diagram from top). The centromeres pass to opposite poles in the succeeding anaphase. This produces a bridge configuration (Anaphase, third diagram from top). If breakage of this bridge configuration occurs at the position of the arrow, a broken chromosome will enter each telophase nucleus (Telophase, right and left, fourth diagram from top). The broken chromosome to the left possesses a duplicated segment, that to the right is deficient for a terminal segment. Continuation of this breakage-fusion-bridge cycle in succeeding nuclear divisions may result in the production of chromosomes with various duplications, deficiencies or duplications plus deficiencies as illustrated in the diagrams below each of these telophase chromosomes. (From McClintock 1941a, through the courtesy of *Genetics*.)

Plants heterozygous for an inversion which does not include the centromere will give rise to altered chromosomes following a crossover within the inverted segment. It is well known that this results in the production of a dicentric chromatid and an acentric fragment. Passage of the two centromeres of the dicentric chromatid toward opposite poles in the meiotic anaphase spindle produces a chromatin bridge configuration. Rupture of this bridge occurs either before or following the formation of the cell plate. The position of rupture varies. It may be adjacent to one centromere or at any position between the two centromeres. In all cases, a ruptured chromatid possesses a deficiency because the dicentric chromatid itself is deficient for a segment carried by the acentric fragment. If the position of breakage in the bridge configuration is non-median, the broken chromatid entering one nucleus will be

meiotic anaphase, fusions occur at the position of breakage between the two sister halves of this broken chromatid (Prophase, second line, fig. 1). As the centromeres of the terminally fused sister chromatids pass to opposite poles in the following mitotic anaphase, a chromatin bridge configuration is produced (Anaphase, third line, fig. 1). As the centromeres approach the poles of the spindle figure, tension is exerted on this bridge causing it to rupture. The rupture may occur at any position between the two centromeres. Following a non-median rupture of the bridge configuration (arrow, upper Anaphase, fig. 1) the broken chromosome entering each sister telophase nucleus will differ in chromatin constitution (upper Telophase, right and left, fig. 1). The behavior of the broken end in each daughter nucleus is similar to its behavior in the previous mother nucleus. Following reduplication of

the chromosomes, fusion occurs between the two sister chromatids at the position of the last breakage and a bridge configuration is produced in the succeeding anaphase which is followed by rupture and the inclusion of a chromosome with a broken end in each sister telophase nucleus. If this process continued, each succeeding mitosis would possess an anaphase bridge configuration because each preceding telophase nucleus had received a chromosome with a broken end. The continuation of this breakage-fusion-bridge cycle should produce chromosomes with various deficiencies, duplications and reduplications of segments following non-median rupture of the bridge configurations in successive anaphases. This subsequent behavior is illustrated in Figure 1. Thus, the production of a dicentric chromatid following crossing-over at a meiotic prophase may initiate a breakage-fusion-bridge cycle. It has been demonstrated that this cycle will continue in all subsequent gametophytic and endosperm mitoses following its origin at a meiotic anaphase (McClintock 1939, 1941a). However, this cycle will cease whenever such a broken chromosome is delivered to the zygote. The broken end heals. This healing is permanent for no further fusions and breakages will occur in the sporophytic mitoses or in any tissues of succeeding plant generations. Because this cycle occurs in the gametophytic divisions (two in the male, three in the female) preceding the formation of the zygote, a wide range of newly organized chromosomes with stable broken ends could be recovered in the sporophytic tissues. These could possess deficiencies of various lengths, duplications of various lengths, deficiencies plus duplications or simple or multiple duplicated segments if the original broken chromosome possessed at least a complete complement of genes of the chromosome.

Although plants heterozygous for an inversion which does not include the centromere produce chromosomes with broken ends at meiotic anaphases following crossing-over within the inverted segment, these inversions usually may not be used to recover chromosomes with modified constitutions because all of these broken chromatids are deficient for a segment of chromatin. In most cases, the deficiency in the genomic complement of the spore receiving a broken chromatid is sufficient to hinder the functioning of the gametophyte arising from it. Consequently, other structural modifications have been used which will produce at meiosis a chromatid with a broken end but with no deficiency of genes of this chromosome. A spore nucleus receiving such a broken chromosome has no deficiency in its genomic complement. A functional gametophyte could be produced from such a spore. Two alterations in the structural composition of chromosome 9 in maize have been used for this study. One is a moderately complex rearrangement of segments composing the chromosome (fig. 2: a, normal chromosome 9; b, rearranged chromosome 9). In the second case, the chromosome 9 possessed a duplica-

tion of the short arm as shown in Figure 3. In each of these two cases, a dicentric chromatid is produced following a crossover between the normal chromosome 9 and the modified chromosome 9 (d, fig. 2; c, fig. 3). If breakage of the dicentric chromatid occurred at or to the left of the arrow in the

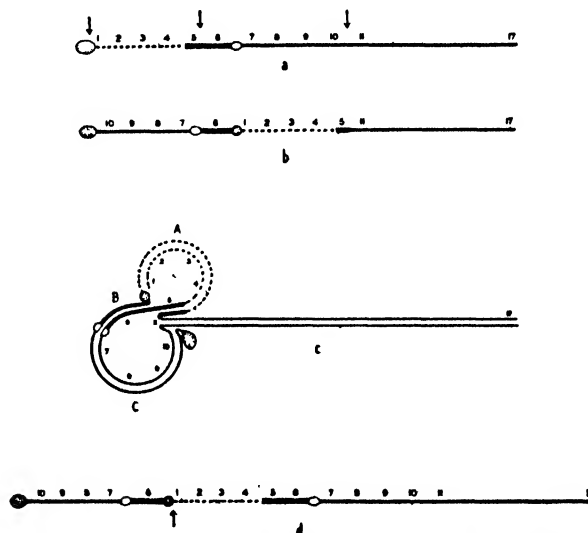


FIG. 2. Diagrammatic representation of a rearrangement in chromosome 9 which may lead to the production of a broken chromosome with at least a full complement of genes for this chromosome. a, a normal chromosome 9. The centromere is represented by the clear oval. The short arm terminates in a large knob. The linear organization of the chromosome is represented by the dash line, the heavy line and the lighter line aided by the numerals. By means of X-rays, the chromosome was broken at the positions of the arrows. Union of broken ends gave rise to the rearranged chromosome 9 as shown in b. The synaptic association of this chromosome and a normal chromosome 9 (with no terminal knob) is shown in c. A crossover in region A will produce the dicentric chromosome shown in d. This dicentric chromosome possesses a full complement of genes of chromosome 9 from the arrow to the right end of the chromosome. A bridge configuration results in anaphase I. If a break occurs at or to the left of the arrow, the broken chromatid to the right will possess at least a full complement of genes of this chromosome. (From McClintock 1941a.)

diagrams, the broken chromatid to the right would possess at least a complete set of genes for this chromosome. The breakage-fusion-bridge cycle which occurs in the subsequent gametophytic divisions could produce chromosomes with various constitutions by the method illustrated in Figure 1. Thus, chromosomes 9 with the various modifications described above could be delivered to the zygote. A wide range of structurally modified chromosomes 9 have been recovered in the progeny of individuals heterozygous for these two modifications (McClintock, 1941a).

It may be seen that the breakage-fusion-bridge cycle is a particularly favorable means of obtaining

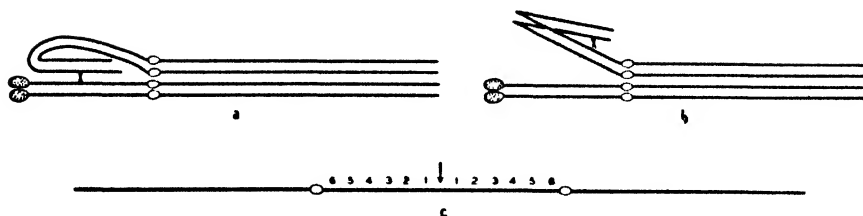
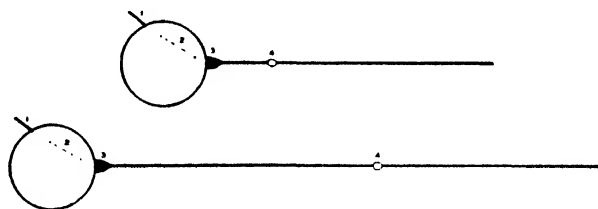


FIG. 3. The synaptic association of a normal chromosome 9 (with a large terminal knob) and a chromosome 9 with a duplication of the short arm in the inverted order (no knob present). The clear oval represents the centromere. A crossover as indicated, following the association in *a*, will produce the dicentric chromosome shown in *c*. Likewise, a crossover as indicated, following the association shown in *b*, will produce the dicentric chromosome shown in *c*. This dicentric chromosome is equivalent to two chromosomes 9 fused at the ends of their short arms. This dicentric chromosome produces a bridge configuration at a meiotic anaphase. If the break occurs at or to the left of the arrow, the broken chromatid to the right will possess at least a full complement of genes of this chromosome. (From McClintock 1941a, through the courtesy of *Genetics*.)

chromosomes with altered sizes and chromatin constitutions. It is not understood why this cycle is confined to the gametophytic and endosperm tissues in the generation immediately following the meiotic origin of the broken end nor why it ceases in the sporophytic tissues and never reappears. The recovered broken chromosome is as permanent in its morphology as any normal chromosome of the complement. The behavior of a chromosome initially broken in the sporophytic tissues is not the same and will be discussed later.

In several dissimilar cases the normal process of crossing-over has been held responsible for alterations in the structural composition of the chromosome. Two of these will be mentioned (McClintock, unpublished). The first case involves the nucleolus chromosome. The appearance of the nucleolus chromosome at prophase is diagrammed in Figure 4. The nucleolus organizer, a deep staining body adjacent to the nucleolus, is responsible for the organization of the nucleolus at telophase (McClintock, 1934). During this process, the segment of chromatin from the organizer to the end of the short arm (the satellite) is removed from the main body of the chromosome by growth of the nucleolus. Although removed some distance from the organizer, it is attached to it by a thread running through the nucleolus substance. If at a meiotic prophase, a chiasma forms between the centromere and the nucleolus organizer and if terminalization of this chiasma proceeds toward the end of the arm of the chromosome, will the terminalizing chiasma stop at the organizer or will it pass through the nucleolus substance to reach the end of the arm? It apparently cannot pass through the nucleolus. The terminalization process either stops at the nucleolus organizer, or, if the force is great enough, the chromatids involved are ruptured at the position of attachment of the organizer to the nucleolus. Following this rupture, fusion occurs at the position of breakage between the nucleolus organizers of the two chromatids involved. Consequently, a dicentric chromosome is formed which results in a bridge configuration at a meiotic anaphase. It will be noted that the segment between the centromere and the



FIGS. 4 AND 5

FIG. 4 (above): Diagrammatic illustration of the normal nucleolus chromosome in maize. The large circle represents the nucleolus. The small, clear oval (4) represents the centromere. The large, deep-staining body (3) attached to the nucleolus represents the nucleolus organizer. The satellite is represented by 1. Because of the growth of the nucleolus at telophase, the satellite is removed from the nucleolus organizer but remains attached to it by a thread (2) which is in or on the nucleolus itself. This condition is maintained from telophase to the following late prophase. If, in a normal plant, a chiasma forms between the nucleolus organizer and the centromere (between 3 and 4) and if terminalization of this chiasma proceeds toward the end of the arm, obstruction occurs when the chiasma reaches the nucleolus. The nucleolus organizers may be ripped from the nucleolus resulting in breakage of the chromatids at this position. Fusion 2-by-2 then occurs between the broken chromatids at the position of rupture. This produces a dicentric chromatid composed of two chromosomes 6 fused at the distal part of their nucleolus organizers. Breakage of this dicentric chromosome at various positions between the centromeres during the following meiotic anaphases produces chromosomes with various modifications in size and chromatin content.

FIG. 5 (below): The nucleolus chromosome in a plant homozygous for a translocation between chromosome 6 and chromosome 5. The description of this chromosome is similar to that given in the legend of Figure 5. The translocation occurred adjacent to the centromere on the short arm of a normal chromosome 6 and toward the end of the long arm of a normal chromosome 5. The centromere of the resulting nucleolus chromosome is located a considerable distance from the nucleolus organizer. Chiasma formation between region 3 (the nucleolus organizer) and region 4 (the centromere) is very frequent in plants homozygous for this translocation. Consequently, the chromatids are frequently ruptured at the attachment of the nucleolus organizer to the nucleolus during terminalization of these chiasmata. Dicentric chromatids are produced following 2-by-2 fusions of ruptured nucleolus organizers.

nucleolus organizer where an effective chiasma could be formed is relatively short in the normal nucleolus chromosome. Relatively few bridge configurations following this process appear at meiotic anaphases. The true nature of these bridge configurations was clearly revealed during a study of meiosis in plants homozygous for a translocation which placed the centromere at a considerable distance from the nucleolus organizer. This translocation chromosome is diagrammed in Figure 5. Chiasma formation is very frequent in the long segment between the centromere and the nucleolus organizer. Many bridge configurations arising from fusions of ruptured nucleolus organizers were observed at meiotic anaphases in these plants. Rupture of the anaphase bridge configurations at various positions between the two centromeres gives rise to chromosomes with variously modified constitutions. As expected, they include various degrees of duplication or deficiency. Their constitutions may be observed readily in the prophase of the following spore divisions. Thus, if chiasmata are the result of crossing-over, as the combined evidence suggests, the normal process of crossing-over may be a factor in the origin of modified chromosomes even when no structural rearrangements are present.

The second case of alteration in the constitution of chromosomes for which crossing-over is held responsible is again related to chiasmata. In several cultures of maize, it has been observed that both the terminal and the interstitial chiasmata in all of the chromosomes of the complement are released or unraveled only with considerable difficulty at the first meiotic anaphase. Consequently, as the disjoining centromeres of the bivalent chromosomes pass toward opposite poles, the chromatin between the centromeres and the chiasma is drawn out into a very fine thread. Frequently the tension becomes great enough to rupture these threads before the chiasma has unraveled and a broken, deficient chromosome enters the telophase nucleus.

From the evidence reviewed, it may be seen that the normal process of crossing-over is a means by which chromosomes with altered constitutions are produced.

ALTERATIONS IN SIZE, FORM AND CONSTITUTION OF CHROMOSOMES FOLLOWING NON-HOMOLOGOUS OR ILLEGITIMATE CROSSING-OVER.

Synaptic associations involving non-homologous parts of chromosomes are regularly present in maize when the chromosome complement is heterozygous for some structural rearrangement or when an unbalanced chromosome complement is present. The nature of this association has been extensively investigated (Burnham, 1932; McClintock, 1932, 1933). The synaptic behavior of the univalent chromosome in monosomic plants or of the extra chromosome in trisomic plants may be used as an example. The non-homologous synaptic associations of the univalent at the meiotic prophase are variable

but one of the most frequent types of associations is diagrammed in *a* Figure 6. This 2-by-2 association is completely non-homologous. Breakage of the chromatin threads and reunions of the broken ends following an illegitimate crossover at $x - x'$ could give rise to a chromosome with an inverted segment or to a deficient rod-shaped chromosome and an acentric ring-shaped chromosome depending upon whether the reunion of the broken ends was diagonal

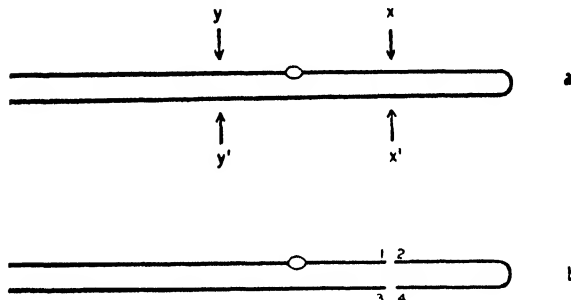


FIG. 6. Diagram representing the non-homologous synaptic association of a univalent chromosome. The clear oval represents the centromere. The chromosome is folded upon itself at the mid-region. The association is completely non-homologous. If an illegitimate crossover occurs at $x - x'$ there are two possible consequences depending upon the resulting 2-by-2 fusions of broken ends. These broken ends are designated 1, 2, 3 and 4 in *b*. The fusion of alternate broken ends, 1 with 4 and 2 with 3, will result in a chromosome with an inverted segment. Fusions of opposite broken ends, 1 with 3 and 2 with 4, will give rise to a deficient rod-shaped chromosome and an acentric ring-shaped chromosome. If the breaks occurred at $y - y'$ in *a*, fusions of alternate broken ends will give rise to a chromosome with an inversion. Fusions of opposite broken ends will give rise to a deficient ring-shaped chromosome possessing the centromere and a deficient, acentric rod-shaped chromosome.

or opposite, respectively (*b*, fig. 6). If the illegitimate crossover occurred at $y - y'$, fusions of diagonal broken ends would produce an inversion while opposite fusions of broken ends would produce a deficient rod-shaped chromosome without a centromere and a ring-shaped chromosome possessing the centromere. With respect to opposite fusions, the deficient rod chromosome could be recovered from the $x - x'$ "crossover" whereas a deficient ring-shaped chromosome could be recovered from the $y - y'$ "crossover." It is interesting to note that just these types of modified chromosomes have appeared in the progeny of trisomic plants. Although no effort has been made to obtain the frequency of these events, the interpretation of their origin as a consequence of illegitimate crossing-over between synapsed non-homologous segments of chromosomes is strengthened by the types of individuals which are occasionally produced in the progeny of plants heterozygous for reciprocal translocations. Heterozygous translocations frequently exhibit extensive non-homologous associations at meiotic prophase. Secondary translocations involving the same

two chromosomes have been recovered from such heterozygous plants. From knowledge of the types of non-homologous synaptic configurations which were known to be present in the parent plant, these secondary translocations may readily be derived on the hypothesis of illegitimate crossing-over.

Haploid plants are characterized by very extensive non-homologous associations. This process may be initiated in some parts of the complement by homologous attractions of unidentified duplicated segments but much of the observed 2-by-2 synaptic association is definitely non-homologous. At the first meiotic anaphase, several of the chromosomes may be associated, 2-by-2, by what appears to be a chiasma. Fragments of various sizes may likewise be present. Both the chromosome associations and the fragments may well arise as the consequence of illegitimate crossing-over between associated non-homologous segments of chromosomes although legitimate crossing-over between homologously associated duplicated segments has not been excluded. As yet, we do not know whether such duplicated segments are present in the complement of maize. A more detailed study of the types of chromosome associations and aberrations at meiosis in the haploid plants or a study of the chromosome complements of the progeny of haploid plants could distinguish between legitimate and illegitimate crossing-over. The legitimate crossovers would be expected to give the same chromosomal rearrangement on a number of independent occasions. On the other hand, illegitimate crossing-over following non-homologous associations would not be expected to occur at the same position on a number of independent occasions. The progeny of haploid plants has not been extensively investigated but it is expected that chromosomes with altered constitutions would appear.

NEW TYPES OF CHROMOSOMES ARISING FROM THE ABERRANT BEHAVIOR OF A TELOCENTRIC CHROMOSOME

It has been suspected for some time, on good observational evidence, that true telocentric chromosomes—that is, chromosomes with strictly terminal centromeres—normally are not present in the chromosome complements of organisms. From these observations, one could conclude that some aberrant behavior of telocentric chromosomes must result in their elimination from the complement or that they become modified in such a way that a true telocentric condition no longer exists. In a recent study by Rhoades (1940) the behavior of a strictly telocentric chromosome has been investigated. The suspicion, based on deductive evidence, that telocentric chromosomes are unstable has been confirmed by this investigation. This telocentric chromosome investigated by Rhoades was discovered in a single plant in the progeny of an individual trisomic for chromosome 5. It was composed of a complete short arm of chromosome 5 with the proximal end ter-

minating in the centromere. No chromatin extended beyond the centromere. Both genetic and cytological evidence indicates that the mitotic behavior of the telocentric chromosome is normal in the majority of mitoses. In some mitoses, however, its behavior must be aberrant. Although these aberrant mitoses have not been observed directly, they may be inferred from the genetic behavior and the types of altered chromosomes which are derived from the telocentric chromosome. Plants were obtained with two normal chromosomes 5, each carrying the recessive mutant *bm* (brown mid-rib, located in the short-arm adjacent to the centromere), and a telocentric chromosome carrying the dominant allele, *Bm*. Variegation for *Bm* and *bm* appeared in some of these plants. It was concluded that this variegation was related to aberrant behavior of the telocentric chromosome which either eliminated the telocentric chromosome from some nuclei or eliminated the *Bm* locus from this chromosome. In several cases, a *bm* sector extended into the tassel. This allowed a cytological determination to be made of the chromosome complement of such a sector. In one such case, the observations showed that the telocentric chromosome had been completely eliminated from the nuclei of the sector. In four other such cases, the original telocentric chromosome had undergone considerable modification. In two of these cases, the telocentric chromosome had been modified and reduced to a small fragment with a *subterminal* centromere. In a third case, a telocentric chromosome was present but its size was only one-half that of the parental telocentric chromosome. In the fourth case, a minute fragment was present composed of only two or three chromomeres and a terminal centromere. Although the observational evidence is insufficient to indicate the methods of origin of these modifications of the original telocentric chromosome, it does indicate that telocentric chromosomes are unstable. They may be eliminated totally from the nuclei or they may produce variants with decidedly altered constitutions.

One recurring type of modification suggests the nature of one type of instability of the telocentric chromosome. When a plant containing this telocentric chromosome in addition to the normal complement is crossed by or onto normal plants, three types of plants are expected in the progeny. These are (1) normal diploids, (2) plants trisomic for chromosome 5 and (3) plants carrying the telocentric chromosome in addition to the normal chromosome complement. Such plants appear in their expected proportions when the female parent carried the telocentric chromosome. Pollen grains carrying either an extra chromosome 5 or the telocentric chromosome rarely function in competition with grains carrying a normal chromosome complement. However, an unexpected type of plant appeared in approximately the same relative proportions in the progeny of these reciprocal crosses. These plants possessed an extra chromosome. This extra chro-

mosome was composed of two short arms of chromosome 5 joined by a single median centromere—a true isochromosome. It is known that pollen grains carrying such an isochromosome in addition to the normal complement do not function in competition with normal grains. However, in the case mentioned, sperm nuclei carrying isochromosomes were delivered to egg nuclei.

It is necessary to explain first the origin of this isochromosome and secondly, how a pollen grain may deliver such a chromosome to the egg nucleus. If one assumed that normal reduplication of the chromatin of the telocentric chromosome occurred in some of the mitoses in the plant carrying the telocentric chromosome, which was accompanied by some form of misdivision of the centromere of this chromosome either in the prophase or in the subsequent spindle figure, an isochromosome in addition to the normal complement could enter one telophase nucleus. Under these circumstances, only the normal chromosome complement could enter the sister telophase nucleus. If this occurred during the division of the microspore nucleus, a generative nucleus carrying an isochromosome and a tube nucleus carrying only the normal complement could be produced. It is assumed that the functioning of a pollen grain is controlled by the constitution of its tube nucleus. Thus, a pollen grain could deliver an isochromosome to the egg nucleus if its tube nucleus possessed a normal chromosome complement and its sperm nuclei carried, in addition, an isochromosome. Such misdivision of the centromere of the telocentric chromosome may be one of the factors responsible for the *Bm—bm* variegation mentioned above.

The evidence reviewed indicates that the telocentric condition is another factor leading to the production of a wide range of spontaneous chromosome alterations.

THE RELATION OF CHROMOSOME FORM TO CONSTANCY OF CHROMATIN CONSTITUTION

In the previous sections it was pointed out that permanency of the constitution of a chromosome will not be maintained through successive nuclear cycles if the chromosome possesses an unstable broken end or if it possesses a strictly terminal centromere. Extensive and varied modification in the size and genic content of chromosomes arise from these two conditions. There is a third condition which leads to extensive modification of the composition of a chromosome. If a chromosome has the form of a ring rather than a rod, it does not maintain itself unaltered through successive nuclear cycles. Although its form does not change, its chromatin composition is continuously subject to alteration. The ring chromosomes may become enlarged by duplication and reduplication of segments composing the ring or they may decrease in size by deletions of segments from the ring. Alteration after alteration will occur if the ring *form* of the chromo-

some is maintained. A plant possessing a ring-shaped chromosome may be a complete mosaic of altered ring-shaped chromosomes. In some cells and tissues the ring chromosome may be deficient for segments of various lengths. In other cells and tissues, the ring chromosome may possess duplications or reduplications of segments. In still other cells and tissues, the ring chromosome may possess both deficient and duplicated segments. The size of the ring chromosome is no indication of its genic content.

Observations of somatic mitoses have indicated the method by which alterations in the chromatin constitution of the ring-shaped chromosome arise (McClintock, 1932b, 1938a, 1941b). It is related to the mitotic cycle. At some mitotic prophase, the two sister halves of a divided ring-shaped chromosome form a continuous, double-sized, dicentric ring chromosome instead of two freely separating, monocentric ring chromosomes (Prophase, fig. 7). This condition could arise subsequent to reduplication of the chromonema of the ring chromosome if a somatic crossover occurred between the two sister chromatids, or the reduplication process itself could lead to this condition. At early anaphase, the two centromeres of the double-sized, dicentric ring chromosome move toward opposite poles of the spindle figure (Anaphase, fig. 7). Tension on the chromatin strands between the two centromeres in late anaphase or early telophase causes them to rupture. The position of rupture is variable. Three such possible positions are indicated by the dash lines *a*, *b*, and *c*, respectively (Anaphase, fig. 7). The subsequent behavior of the broken strands is illustrated in the bracketed figures (lower row, fig. 7) for each of these breakages. Segments of the broken double-sized ring chromosome enter each telophase nucleus but the chromatin composition of the segment in the sister telophase nuclei may differ considerably. In each telophase nucleus, fusion occurs between the broken ends of the segment thus reestablishing the ring *form* of the chromosome but not necessarily its original chromatin and genic composition (Telophases, fig. 7). It may be seen that ring chromosomes with duplicated segments of ring chromosomes with deficient segments may be produced by this process. Repetition of this process in a later mitosis could give rise to ring chromosomes with reduplicated or multiple segments of the original ring chromosome, to ring chromosomes with still greater deficiencies or to ring chromosomes with deficiencies plus duplicated segments. The frequency of occurrence of these aberrant mitoses depends on the length of the chromonema of the ring chromosome—the longer the chromonema the more frequent the aberrant mitoses. The aberrant mitotic configurations may occur in twenty percent of all mitoses if the chromonema composing the ring is as long as the longest chromosome of the normal complement. If the ring chromosome is only one-tenth of this size, an aberrant mitosis may occur in only one percent of the mitoses. If the ring chromosome

is only one twenty-fifth of the size of the longest chromosome, an aberrant mitosis may occur in only 0.2 percent of the mitoses. In all other nuclear divisions, the behavior of the ring chromosomes is normal; the two sister halves of the ring chromosome separate freely at anaphase along with the rod chromosomes of the complement.

segments of the dicentric ring chromosome might likewise heal and become stable.

CONCLUSIONS

Permanency of chromosome form and constitution through successive nuclear cycles is a basic postulate of genetic theory. This postulate is well

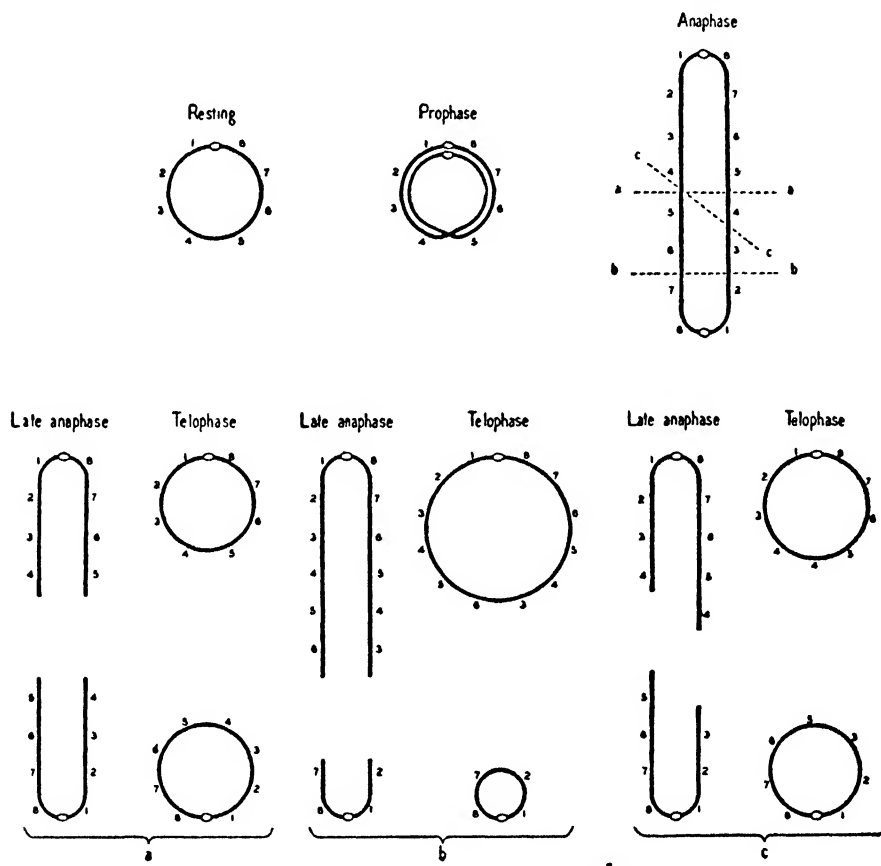


FIG. 7. Diagram illustrating a method by which a ring chromosome becomes altered in chromatin constitution. Upper left: A ring chromosome in a resting nucleus. The clear oval represents the centromere. The individual parts of the ring chromosome are designated by the numerals. Upper middle: A prophase configuration following a "crossover" between the two sister chromatids of the divided ring chromosome. A dicentric, double-sized ring chromosome is produced. Upper right: Appearance of the dicentric ring chromosome in the following anaphase. Breakage of the chromatin strands between the centromeres may occur at any position. Three possible positions *a*, *b* and *c*, respectively, are indicated by the dash lines. The resulting broken strands at late anaphase and the new ring chromosomes formed at telophase by fusions of broken ends of these strands are diagrammed below in the bracketed figures for the breaks *a*, *b* and *c*, respectively. (From McClintock 1941b, through the courtesy of *Genetics*.)

It should be emphasized that fusion of broken ends apparently always follows the breakage of a double-sized, dicentric ring chromosome during an aberrant mitosis in the sporophytic tissues. Although extensively looked for, no cases have been found where the broken ends had failed to unite. Since it has been proved (McClintock, 1941a) that a single broken end which is unstable in the gametophytic tissues may heal and become permanently stable in the following zygote or early sporophyte, it is expected that under certain conditions which at present are not known, the broken ends of the

founded on extensive observational evidence in a wide range of organisms. Knowledge of conditions which will produce changes in this constancy has been of utmost importance in recent years. X-rays, ultraviolet radiation, heat, aging, etc., have been the usual agents producing these desired conditions. It has been known for a long time that other conditions may lead to changes in the form and constitution of chromosomes. When the previous history is not known, the observed changes would naturally fall under the heading of spontaneous aberrations because the conditions responsible for their

occurrence were not apparent. In this discussion, I have attempted to indicate the extent of our knowledge in maize of the conditions which are responsible for such "spontaneous" aberrations. We do know that they may occur (1) under genic control, (2) following legitimate crossing-over, (3) following illegitimate crossing-over, (4) during or following reduplication of the chromonema of the chromosome, (5) as the consequence of the instability of broken ends of chromosomes, (6) during terminalization of chiasmata and (7) as the result of the aberrant behavior of a strictly terminal centromere.

Studies of spontaneous aberrations in maize have contributed and should continue to contribute to our knowledge of the behavior of chromosomes in general. A few such contributions may be summarized. We know from the study of ring-shaped chromosomes that the reduplication process of a chromosome usually occurs along a single plane. The possibility that some sister strand crossover chromatids may be present at meiosis is likewise suggested by these studies for the double-sized dicentric ring chromosomes represent some form of sister chromatid exchange. Although the method of origin of these exchanges is not known, they may be the result of some process which is shared by all chromosomes. If so, the frequency of sister-strand crossover chromatids at meiosis should be directly proportional to the length of the chromosome. However, the process which gives rise to these strands need not be related to the normal process of crossing-over. We are considerably better informed about the stability of broken ends of chromosomes through studies of the types of modified chromosomes which arise following mechanical rupture of chromosomes at meiosis, and following mechanical rupture of dicentric ring-shaped chromosomes at a somatic mitosis. With regard to this, we know that following mechanical rupture of two adjacent chromatids at a late meiotic prophase, fusion may occur between these two chromatids at the position of breakage. However, if these two adjacent chromatids are broken at the first meiotic anaphase, fusions will now occur at the position of breakage between the two sister halves of each of these chromatids. Likewise, if a single chromatid is ruptured at a meiotic anaphase, fusion will occur at the position of breakage between the two sister halves of this chromatid and thus initiate the breakage-fusion-bridge cycle which characterizes the behavior of this chromosome in subsequent gametophytic and endosperm tissues. We know also that fusions may occur between two broken ends of a single chromosome if this single chromosome has suffered mechanical rupture at two points during anaphase of a mitotic division in the sporophytic tissues. From these studies we have determined that a recently broken end of a chromosome is unstable in certain tissues and under certain conditions but may become completely and permanently stable under

other conditions. Because of these observations, we know why ring-shaped chromosomes and telocentric chromosomes cannot maintain themselves in nature and thus why they are not frequently encountered. Through the study of the composition of altered chromosomes arising from haploids we may be able to detect the presence of possible duplicated segments in the normal complement of maize which at present are undetected but suspected from genetic evidence. Even the concepts of chiasmata formation and terminalization may be illuminated through the studies of specific chromosome alterations.

It is obvious that the changes in size, form and constitution of chromosomes have made it possible to detect and study some of the processes underlying chromosome behavior in general.

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DISCUSSION

MULLER: What is the rate of apparent "gene mutation" in the sticky stock?

McCLINTOCK: It is very high, although the homozygotes are very low in fertility. The progeny may also possess chromosomal rearrangements.

GATES: How high is the pollen sterility?

McCLINTOCK: Very high. Many plants shed no pollen at all.

BOCHE: Would incomplete division be an alternative to the refusion of broken ends?

McCLINTOCK: We cannot distinguish by our method between incomplete division or fusion following division. All we know is that the two sister chromatids are fused at the position of previous breakage.

MICKEY: Do bridge chromosomes break with greater frequency at one position more often than at any other position?

McCLINTOCK: Yes, they tend to break at the position of previous fusions.

HUSKINS: If this point were near the middle, the effect could be mechanical.

McCLINTOCK: Yes, but the break would not be expected to be exactly in the middle, although approaching it.

FANO: Is crossing over involved in the behavior of the ring chromosomes?

McCLINTOCK: I think somatic crossing over between sister chromatids may be responsible for the double-sized, dicentric ring chromosomes.

MULLER: If a plant is heterozygous for the ring, can such crossing over occur within the ring?

McCLINTOCK: Yes.

SCHULTZ: Possibly evidence might be obtained from this for sister-strand crossing over at meiosis in maize, which would probably then be more frequent than in *Drosophila*.

McCLINTOCK: The behavior of ring chromosomes suggests some form of sister-strand exchange and also suggests that the frequency with which it occurs would depend on the length of the chromonema composing the chromosome. The longer the chromonema, the more frequent the expected occurrence.

NEBEL: Changes in size might also be due to a twist in the plane of reduplication within the ring.

McCLINTOCK: Yes, it is possible. However, the evidence suggests that this must be infrequent.

MICKEY: Will a gene such as sticky increase the frequency of double sized rings?

McCLINTOCK: There is no evidence for this in our experiments, but no actual measurements of the frequency in different strains have been made.

SANSOME: Your ring chromosomes came from X-ray treatment, didn't they?

McCLINTOCK: Not in all cases. Ring chromosomes may arise following X-radiation but also spontaneously.

DELBRÜCK: Do sister-strands heal following breakage of a dicentric ring chromosome?

McCLINTOCK: I have not found such a case. Such healing would give rise to a rod-shaped fragment. They have been looked for but have not been found. Since broken ends are known to heal under certain as yet unknown circumstances such rod-shaped fragments arising from broken ring chromosomes eventually may be found.

SPARROW: If we assume that the ring chromosome is longitudinally bipartite and that it breaks at anaphase, sister-strand crossing over as shown in the diagram is not necessary to explain double or interlocked rings.

McCLINTOCK: The diagram represents the simplest interpretation for this presentation.

MULLER and GLASS (simultaneously): Do you get interlocked rings?

McCLINTOCK: Because of the small size of the mitotic chromosomes in maize, interlocking of two sister ring chromatids would be difficult to detect. At anaphase they would look like a twisted double sized ring; this configuration is frequent but one cannot be sure that it represents interlocked ring chromatids. Interlocked ring chromosomes occur following another type of behavior of ring.

CARLSON: When there is a break followed by refusion between the nucleolus organizer regions, where does the nucleolus form in the daughter cells with reference to the nucleolus organizer?

McCLINTOCK: Development of the nucleolus takes place at the position of maximum activity of the two fused organizers, which is at the position of fusion of the two organizers.

WARMKE: Concerning the mechanism of bridge formation involving the nucleolus chromosome, if terminalization occurs late, after the nucleolus has disappeared, then does the nucleolus organizer itself inhibit terminalization and thus cause the break?

McCLINTOCK: Probably not. The breakage is assumed to occur before the nucleolus disappears. The frequency of bridges is low in proportion to the chiasmata frequency or crossing over within this region.

WARMKE: In *Datura*, crossing over apparently regularly occurs between the centromere and nucleolar organizer, and terminalization takes place with the formation of what have been called humps; chiasmata form without breaks in this case.

McCLINTOCK: In maize, when the distance between the nucleolus organizer and centromere is short, there are few bridges. As this distance increases, the frequency of the bridges increases. An increase in the distance is obtained from homozygous translocations.

INDUCED CHROMOSOMAL BREAKS IN DROSOPHILA

B. P. KAUFMANN

Determination of the nature of chromosomal alterations may be based on examination of changes occurring in nature or of those which arise spontaneously or are induced experimentally. Although the changes existing in natural populations of *Drosophila* are more widespread and diversified than was formerly suspected (see review by Dobzhansky, 1941), they obviously represent a residual group which has survived the control of natural selection. Thus inversions form by far the largest store of types detectable in these natural populations, although translocations as well as inversions are known to arise both as a result of X-ray treatment and spontaneously. Chromosomal alterations that have occurred in the phylogeny of *Drosophila*, as inferred through comparisons of the gene arrangements of related species, consist also largely of inversions, combined with rearrangements involving entire chromosome limbs and only very few short interstitial translocations. No translocations of any kind have, however, been found in natural populations within any species of *Drosophila*. Spontaneous chromosome breakage in these insects appears to be very infrequent (Dobzhansky, 1941). This situation contrasts with that observed in certain other organisms, particularly plants, in which spontaneous breaks are not uncommon.

In *Drosophila*, therefore, a profitable analysis of the nature of types of chromosomal changes must depend on a more diversified store of material than is provided in nature. Since Muller's discovery (1927) of the efficacy of X-radiation in increasing the rate of mutation, radiations of various kinds have been employed in producing gene and chromosomal alterations. The legitimacy of comparisons between induced and spontaneous changes may well be questioned. An inquiry into the nature of the reaction of the living system to tools of known physical properties is but one step in the detection of the processes leading to an understanding of their natural counterparts. However, continuation of this method of attack on the problem seems warranted by certain striking similarities between the spontaneous and induced changes. Thus in *Drosophila*, Slizynski (1938) found no primary difference between lethals produced in these two ways; Demerec and Fano (1941) noted the similarity in frequency distribution of both types among the "single event" changes at the Notch locus. Among plants, in *Tradescantia*, according to Giles (1940), and in *Allium*, according to Nichols (1941), chromosomal changes are similar with respect to type and position whether induced or spontaneous. Some contrary evidence has been presented. Helfer (1941) has found that induced breaks in *D. pseudoobscura* are distributed among the chromosomes at random and are not ac-

cumulated in the third chromosome as are the breaks recorded in the inversions found in nature. Stadler (1932) has suggested that X-ray induced mutations in maize appear to be deficiencies and therefore different from the naturally occurring genovariations.

An understanding of the methods by which chromosomal alterations are produced is an integral part of the gene problem, since the phenomenon of position effect testifies that genetic effects may accompany chromosomal alterations or be associated with them. Viewed from one extreme chromosomal changes form the crux of the gene problem, for the view has been presented that all gene changes may embody some rearrangement of chromosomal materials. However, Slizynska (1938) has shown that mutational change need not be accompanied by any perceptible alteration in the salivary gland chromosome configuration. If in these cases any position effect is involved, it must be due to a submicroscopic rearrangement. At this level, as Sax (1938) has pointed out, the distinction between position effect and mutation becomes purely arbitrary.

Detection of induced chromosomal breaks may rely upon either genetic or cytological methods. The genetic techniques, first developed for use with *Drosophila*, utilize suitable chromosome markers so as to permit recognition of the new linkage relationships established by an exchange of chromosome sections. These methods have been widely explored in pursuing questions relating to the architecture of the chromosome and to quantitative studies of dosage relationships (see review by Dobzhansky, 1936). In other experiments, breaks in certain localized regions have been detected by the position effect phenomenon, or alteration in phenotype which accompanies displacement, using such markers as cubitus interruptus, dominant alleles of brown, and in a more restricted fashion Bar, and roughest⁸. The position of the break within the physical chromosome can be plotted with some degree of accuracy by use of such topographical features as the constrictions which are best seen at late prophase and metaphase in somatic mitoses. Although this method has been largely displaced by the salivary gland chromosome technique, it remains a necessary adjunct to critical studies involving the Y-chromosome and the heterochromatic regions of other chromosomes which are relatively inconspicuous in the polytene state. The customary procedure in salivary gland chromosome analysis includes irradiation of the adult male, mating it with a non-irradiated female, and examination of salivary glands of larvae of their F₁ offspring. Cytologically detectable changes induced in the chromosomes contributed by the irradiated father can be recognized in the gland

cells by comparison with the normal, wild-type maternal chromosomes. Treatment of sperm in the male permits considerable plasticity in laboratory practices; males may be stored for several days prior to mating or they may mate repeatedly during the period covering at least the first 12 days after treatment without exhausting the sperm which was mature at the time of irradiation (Demerec and Kaufmann, 1941).

The genetical and cytological techniques for detecting chromosome breaks have each certain limitations and restrictions that must be considered in appraising their merits. Selection of F_1 larvae and removal of their glands for chromosome studies eliminates as potential stocks any induced alterations that might have proved useful for further analysis. Growing of larvae for adequate cytological preparations demands optimum culture conditions; microscopical inspection of the slides proceeds so slowly as to preclude the compilation of massive data. Moreover, the single pair of salivary glands which this method provides may not be adequate for critical analysis. In some of these respects the genetic methods based on the inspection of adults offer advantages. On the other hand, certain types of changes seem to act as lethals in the pupal stages, others may lead to sterility and would not therefore be available for genetic analysis. A greater advantage of the cytological method lies in the opportunity offered for the detection of all breaks, except those restricted to heterochromatin, so that the more complex multiple-break rearrangements need not be confused with the simpler ones. These advantages, coupled with the possibility of localizing each break accurately, are unique to the salivary gland chromosome type of analysis, and have recommended its use in the studies now to be described.

The Validity of the Method for Quantitative Work

The F_1 third instar larvae derived from an irradiated father will show in their salivary glands either an unchanged or an altered set of chromosomes of paternal origin. Determination of the ratio of these two types is the basis measurement in quantitative studies. It is important therefore to attempt to discover to what extent any selected sample furnishes a measure of the proportions in which the altered sperms are produced. No direct answer is possible because the detectable types are but the residue of those originally produced. Assuming the functional capacity of sperms containing deranged chromosomes, for which there is sufficient experimental evidence, a considerable portion of the induced changes must be eliminated as dominant lethals during the embryonic stages. Sonnenblick (1940) has presented cytological evidence showing that badly disturbed mitotic figures may occur in the embryonic stages of the progeny of irradiated parents, but until more adequate data of a quantitative nature are presented, it can only be assumed that the dominant lethals result from distribution of dicentric and

acentric chromosomes as well as more complex, unbalanced types. Even the apparently unaltered sets may contain some chromosomes in which breaks or potential breaks induced by the irradiation have "healed" to restore the original sequence of banding. The contributions of these different types to the fraction of glands which are available for study have been considered by Catcheside (1938) and Bauer (1939), and will be treated more extensively in another paper of this symposium by Dr. Ugo Fano.

It is apparent therefore that in the absence of more satisfactory criteria we must rely upon the homogeneity of successive samples given identical treatment to evaluate the validity of our methods. It has been found that within the range of dosages used in most experiments (up to 4000 r) the proportions of altered and unaltered sperms do not vary significantly from day to day during the period that the larvae are being selected. At 5000 r Bauer (1939) found a significantly higher frequency of aberrations in the second half of the preparations secured than in the first half, suggesting that at this dosage the complex rearrangements lengthen appreciably the embryonic and larval periods. Samples secured from different treatments at the same dosage have been compared for homogeneity by Bauer. They are in general quite uniform, although occasionally a group is secured that deviates widely from the mean. A satisfactory explanation of this phenomenon has not been reached. It is obviously not always an error of dosimetry but apparently some undetected biological variability. Elimination of such variants has occasionally been made in experiments within the lower dosage range (1000-5000 r) without distorting the slopes of the curves involved; at higher dosages (12,000 r) this variability constitutes a considerable and at present unsolved problem.

Distribution of Breaks in the Chromosome Set

Single breaks, such as might lead to terminal deficiencies, are rarely preserved in the salivary gland chromosomes of individuals surviving to the late larval stages. The positions of the two or more breakage points which are therefore discernible in each set of altered chromosomes are affected by the amount of recombination occurring between different regions of the chromosomes. However, the pool of all breaks which are detectable offers an index of the relative fragility of different chromosome regions plus the average ability of a break in a particular region to combine with any other break or group of breaks.

Determination of the positions of more than 600 breaks in the X-chromosome of *D. melanogaster* (Kaufmann, 1939b) has shown that there are no long regions immune to induced breakage, since the breaks were distributed among 111 of the 114 euchromatic subdivisions of Bridges' 1938 map. Localization to subdivision has been reported only for the X-chromosome, but more than 2000 breaks

which were plotted by Bauer, Demerec and Kaufmann (Kaufmann and Demerec, 1937; Bauer, Demerec and Kaufmann, 1938; Bauer, 1939) are distributed among all the divisions of all the chromosomes of this species. Each of the limbs of the V-shaped autosomes and the long arm of the X have approximately equal numbers. These limbs are similar in length. The small fourth chromosome reveals few breaks. The Y-chromosome, somewhat longer than the X in metaphase figures of somatic mitoses may show a somewhat lower break frequency than the autosome limbs. This is partly attributable to the fact that the Y is so inconspicuous in the salivary gland nuclei that only translocations can be detected, the inversions which form a high percentage of observed changes in other chromosomes remaining indiscernible. From these data it is apparent that break distribution among the chromosomes is approximately proportional to chromosome length. Similar evidence has been presented by Helfer for *D. pseudoöbscura* (1941). It is not immediately clear, however, whether break distribution parallels more closely mitotic chromosome length or that registered in the salivary gland chromosome. In the latter the heterochromatic regions, such as most of the Y-chromosome, the proximal parts of the X and of the autosomes constitute a much smaller proportion of the total length than in dividing nuclei. Break frequency in these heterochromatic regions is not correspondingly low but is approximately proportional to the mitotic chromosome length (Kaufmann and Demerec, 1937; Bauer et al., 1938; Kaufmann, 1939b; Bauer, 1939). This similarity has led to the concept that euchromatin and heterochromatin break with equal frequency in proportion to the length which the regions occupy in the chromosomes of dividing cells. The percentage of breaks occurring in the proximal heterochromatic regions of the different chromosomes parallels closely the proportion of heteropycnotic chromatin seen in these chromosomes during the early prophase stages in mitosis. Bauer has pointed out that the values obtained may be somewhat low because of undetectable breaks. On the other hand, errors in the opposite direction may be introduced, since the limits of the proximal heteropycnotic regions seen in mitotic cells have never been established with precision on the salivary gland chromosome maps. If the proximal divisions of these maps, generally regarded as delimiting heterochromatin, should contain some euchromatin, as now seems possible (Mr. Taylor Hinton, unpublished) an undetermined but probably small percentage of euchromatic breaks have been included in our determinations of break frequency in heterochromatin.

As an alternative to the explanation of proportional break frequency, Muller and co-workers (1937) have suggested that there are only a few loci in the heterochromatin which must therefore be subject to breakage with a much higher frequency than euchromatin loci. The cytological details of this

analysis which requires the precise location of different breakage points within a section of the prophase or metaphase X-chromosome about one micron in length, have unfortunately never been published.

There are also certain intercalary regions with high break frequency that may occupy a proportionately greater length in mitotic than in salivary gland chromosomes. Several of them have been located in the X-chromosome of *D. melanogaster* (Prokofyeva-Belgovskaya and Khvostova, 1939; Kaufmann, 1939b). Those that break most freely have unique cytological properties; they appear as reversed repeats (the *abccba* sequence of banding) and often pair with each other or the proximal heterochromatin. This behavior suggests that they are heterochromatic regions which may have arisen in phylogeny by repeated inversion and duplication within the chromosome. Such regions probably occur along the limbs of all the chromosomes, as judged by observed pairing relationships, but their distribution has not been measured by the break frequency method.

Further departures from random break distribution as measured in the salivary gland cells have been reported for the terminal parts of chromosomes, but high values obtained for these regions are probably attributable to the greater capacity for recombination due to their positions. In *D. melanogaster* the distal divisions of all the long chromosomes show an increased break frequency as compared with the more proximal divisions. Among 475 breaks plotted within the 19 euchromatic divisions of the X-chromosome (Kaufmann, 1939b), 30 were in division 1 as compared with the 19.13 expected on the basis of the length represented. The probability of chance giving this type of distribution lies between .01 and .02 when measured by the χ^2 test. In this experiment the greatest deviation from expectancy was in section F of division 1 and not in the more distal subdivisions, as might be expected if the increase were attributable to heterochromatin terminally located, as suggested by Prokofyeva-Belgovskaya (1938). From the extended data of Bauer's study (1939) there appears to be a slight but gradual increase in frequency which proceeds distally along the euchromatic portion of the chromosome, with a pronounced rise in the terminal section. In further studies of break distribution in different parts of the chromosome (Kaufmann, unpublished) a comparison was made between the number of breaks in chromosomes with the delta 49 inversion and those having the wild-type sequence of banding. Within the limits of the inverted section (4D to 11F) there was greater similarity when regions were compared on the basis of their identicalness than when compared on the basis of distance from the tip (for the former a value of P between .2 and .3 was obtained by the χ^2 test; for the latter a value between .05 and .1). Similar studies are now in progress measuring break frequency in vari-

ous displaced sections, including tip regions of chromosomes of considerably different lengths which have arisen by reciprocal translocation.

These departures from random break distribution, whether attributable to intercalary heterochromatin or to greater freedom of recombination, although statistically significant, are nevertheless small. The intercalary regions of high break frequency are probably distributed fairly uniformly throughout the chromosome set if we may judge by the close correspondence between break frequency and chromosome length. A further test of the general randomness of break distribution is provided by measurements of the lengths of inversions and the distance of breakage points from the centromeres in reciprocal translocations. It should be possible from such measurements to determine whether a break in one division has an equal opportunity of combining with a break in any one of the other subdivisions. Comparison of observed lengths of these rearrangements with those expected on random distribution are extremely close (Bauer, et al., 1938; Bauer, 1939; Helfer, 1941). Application of the method to the 3-break rearrangements (Bauer, 1939) has likewise demonstrated that break distribution is essentially at random.

In considering, however, the proportions of different types of rearrangements, such as inversions and reciprocal translocations, as a measure of the participation in recombination of breaks in the different chromosomes, certain departures from randomness are apparent. If the chromosome ends resulting from a break in one of the five longer chromosome limbs (2L, 2R, 3L, 3R, X) have an equal opportunity to combine with ends resulting from a second break in the same or in any other limb, inversions should be one-fourth as frequent as reciprocal translocations. This expectancy is based on the assumption that when two breaks occur at random they will fall in the same chromosome limb in one-fifth of the cases. It is assumed, moreover, that four broken ends with opportunities for combining in all possible ways will give the same proportions of viable rearrangements whether both breaks are in the same or in different limbs (Catcheside, 1938). In one type of combination the breaks may heal to restore the original sequence of banding, so that chromosomes appear cytologically unchanged (if one of the two breaks is able to heal independently of the other the result will be a non-viable terminal deletion, as if only one break had occurred). A second possible pattern of recombination leads to the formation of a non-viable alteration, either a dicentric or ring chromosome combined with an acentric fragment if the breaks are in different limbs, or a deficiency and a fragment if both breaks are in the same limb. The third possible kind of combination gives the detectable alterations, inversions or reciprocal translocations. The observed ratio of the latter types is 1 to 2, that is the inversions are twice as frequent as expected. There

seems no evidence for assuming that the formation of inversions from the original broken ends is favored by the failure of production of the non-viable combinations or that more inversions survive because selection operates against the translocations. It seems more reasonable to conclude that when a break occurs in a chromosome limb it has a greater opportunity of combining with other broken ends if they are in the same limb than if they are in other limbs. Bauer (1939) has interpreted this to imply a restricted recombination zone such as might be imposed by spatial limitations in which only three of the five chromosome arms could participate.

Such an interpretation is not supported by data from the 3 and 4-break rearrangements. In them observed break distribution corresponds closely with values expected on the assumption that all five chromosome limbs are free to participate in recombination. Determination of the expected values in multiple-break cases depends not only on the original break distribution among the chromosomes but also the proportion of viable survivors. Thus, in 3-break rearrangements the 1,1,1; 2,1; 3 types will be produced by random distribution in the ratio of 12:12:1, but viable combinations will be half as frequent in the 1,1,1 distribution as in the other two, so that the expected ratio among the survivors is altered to 6:12:1.

Another measure of the recombination process is the number of independent rearrangements produced within the irradiated nucleus. With only two or three breaks participating not more than a single detectable new combination can result. But four or more breaks afford opportunities for concurrently existing independent rearrangements. With four breaks the single complex rearrangements involving all breaks (designated as 4) are expected to appear about five times as frequently as two independent rearrangements (designated as 2 + 2), if it is assumed that breaks occur at random and that non-viable combinations are eliminated. Observed frequencies, however, are distinctly in the opposite direction. Different experiments show between four and ten times as many of the 2 + 2 combinations as the 4 (Bauer, et al., 1938; Bauer, 1939). It seems probable that propinquity favors recombination in pairs; that broken ends can meet and form new attachments without interference from more remote breaks. This pattern of recombination probably accounts for the preponderance of even-numbered-break rearrangements over those with odd numbers of breaks as noted by Fano (this volume). There is no evidence, however, of a restricted sphere of recombination, since as many as seven to nine breaks have been observed to participate in a single rearrangement (Kaufmann, 1939a; Dr. E. Sutton, unpublished). The proportions of the various recombination types at different dosage levels have been considered in detail by Fano (this volume).

It had generally been assumed that new combina-

tions were established within a short time following irradiation, and on this basis certain opinions have been presented concerning the disposition of the chromosomes within the sperm head. The question of whether restitution occurs shortly after treatment can be tested by comparing the effects of equivalent doses of X-rays, the one administered continuously, the other in a series of fractions. Experiments of this kind have been conducted by Muller and Makhijani and by Kaufmann. Muller and Makhijani (Muller, 1940) found by genetic methods that equivalent percentages of translocations between the second and third chromosomes were induced by dosages of 1500 roentgens given to impregnated females, whether in a single treatment with immediate or delayed opportunities for egg laying, or in a series of four fractions. The results indicate that breaks induced during the first fractions of a series do not heal in the interval between the treatments, for if they did these broken ends would not be available to participate with those subsequently produced in the formation of chromosomal aberrations. The salivary gland chromosome method of analysis utilized by Kaufmann (1941) affords additional criteria for comparing the two methods of treatment. Total break frequency can be determined as well as the percentage of altered sperms. Analysis of the different kinds of aberrations will indicate whether the multiple-break, complex rearrangements are reduced in frequency or eliminated in the fraction series as would be expected if recombination were occurring in the interval between successive treatments. Furthermore it seems possible on a priori grounds that if recombination occurred during these intervals inversions induced in the later fractions might be superimposed on those produced by previous treatment to give the overlapping and included types. Fraction treatments of males were spaced at 24 hours in the earlier experiments and at 16 days in the later ones. Following both schedules the kinds and proportions of changes recorded in the salivary gland chromosomes were in close agreement with those found in the controls; there was no evidence of restitution or recombination, no elimination of the complex rearrangements, no overlapping inversions. This evidence, although indirect, indicates clearly that breakage and reunion do not occur simultaneously as part of a single process. In the sequence of events the physical and chemical action of radiation (subsequently referred to as "ionization") distributed at random produce breaks or potential breaks within the chromosomes of the sperm head. Subsequently the union of broken ends is favored by their propinquity; only in this way is a contact mechanism prerequisite to the production of rearrangements.

Since recombination is not effected within the period of 16 to 28 days measured in these two independent experiments, it is quite unlikely that it can occur within the mature sperm. Up to this time efforts to determine the arrangement of the chromo-

somes within the sperm head of *Drosophila* have failed even with the use of the electron microscope. Observations on other insect sperms, including another Diptera (Wolf, 1939), may be interpreted as indicating that the chromonemata are elongated, closely appressed and therefore immobile. It is probable that this immobility is the limiting factor which inhibits recombination, since close contact seems assured within the linear sperm head whose diameter is about the same as that of a single metaphase chromosome. Metz and Bozeman (1940) have likewise concluded that lack of movement is the main factor in preventing chromosome rearrangements following irradiation of oöcytes of *Sciara*.

Movement of the chromosomes is certainly assured after the sperm has penetrated the egg and begun to round up to form the male pronucleus. There are, however, certain intimations that recombination may be delayed until much later. Helfer (1940) has observed a mosaic salivary gland in *D. pseudoöbscura* showing four different types of cells with respect to chromosomal rearrangements. One of the possible explanations of their origin is that reattachment of broken ends was delayed until after the second cleavage division. Sidky (1940) and Helfer (1941) have obtained rearrangements which they interpret as translocations between chromosomes of paternal and maternal origin. In both cases a plausible alternative explanation is that the chromosomes involved were all of paternal origin. Glass (1940) reports that no translocations between chromosomes of maternal and paternal origin were secured following irradiation of impregnated females. Such translocations would furnish critical evidence concerning delayed reattachment. Recombinations of this type must occur subsequent to the formation of the first cleavage spindle upon which the chromosomes of the male and female pronuclei remain separated as independent groups (gonomery). Until more critical evidence is forthcoming it can perhaps be assumed that if chromosome recombination is not realized within the male pronucleus, the breaks or potential breaks may remain capable of reunion for a limited time, during which contacts with other chromosomes may be effected.

If such a long delay in reattachment can occur it seems probable that "ionization" would produce potential rather than actual breaks within the chromosomes of the spermatozoa. Otherwise it is difficult to account for the persistence of broken ends through one or more cleavages without the loss of terminal fragments or without union of sister chromatids to produce dicentric and ring chromosomes. Fusion of chromatids might be prevented in some cells by the temporary action of a set of inhibiting conditions, comparable to those found by McClintock (1941) in sporophytic tissue of maize. Such action would not account, however, for the normal division and distribution of acentric fragments through one or two cleavage mitoses. If ionization should produce only potential breaks

restitution must likewise be inhibited through the first cleavage divisions in any cases in which recombination is so long delayed. A situation may exist in which temporarily no restitution has occurred and during which potential breaks are utilized in combination with other similar regional alterations.

Since the interval between irradiation of the sperms of *Drosophila* and their utilization in fertilization can be extended over many days without break restitution or recombination taking place, an opportunity is offered to attempt to alter these properties experimentally. Various types of supplementary irradiation, such as the near infra-red and ultra-violet, or chemical treatment of sperm, if capable of altering the customary behavior of the X-ray sensitized regions might throw some light on the nature of the disturbances produced in the chromosome by "ionization." Experiments of this kind have been undertaken in cooperation with Dr. A. Hollaender, but since only preliminary data are available at present, a statement concerning the results obtained will be reserved for a later publication.

Chromosome and Chromatid Breaks

The irradiated sperm normally contributes to the cells of the embryo a complete set of chromosomes. A few rearrangements reveal deficiencies. In other alterations the set has been supplemented by short duplicated sections of one or more of the chromosomes, so that these regions appear in triplicate in the salivary gland nuclei. The frequency with which duplications are detected is very low; Bauer (1939) reports 7 among 688 mutated sperms. Eight additional cases have been found in material analyzed by the writer, occurring with about the same frequency.

Since the duplications involve two strands contributed by one parent, they constitute valuable material from which inferences may be derived concerning the structure of the chromosomes of the mature sperm at the time of irradiation. The suggestion that chromosomes might be longitudinally double at this stage was advanced by Patterson (1933) from a study of the effects of X-radiation on the production of mosaic flies by breaks in the X-chromosome. Subsequently, Moore (1934), White (1935), Carlson (1938), Sax (1938), Kaufmann (1938, 1939a) have emphasized the possibility that in *Drosophila* and other organisms the effect of a single ionization might result in the breakage of two adjacent strands, probably through severing of some retaining or sheathing material such as a chromosome matrix. Attempts have been made to refute and discredit this view largely on theoretical grounds. A considerable mass of data from irradiation experiments has been appraised with respect to the time of chromosome division, using as a criterion the postulate that if a chromosome is split, a single ionization will affect only one of the two strands to produce a chromatid break, whereas a chromosome

break induced in an undivided chromosome will be discernible in each of the two strands subsequently produced by longitudinal division. Duplications are useful material for studying this problem.

Seven chromosome breaks were detected among the eight duplications analyzed by the writer (fig. 1). They are apparently distributed as much at

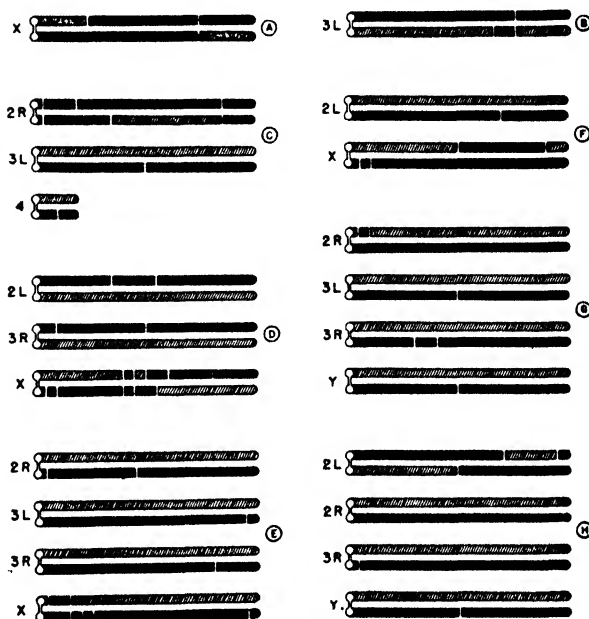


FIG. 1. Chromosome and chromatid breaks—Analysis of positions of breaks in eight rearrangements which showed duplicated sections of one of the chromosomes involved. Chromosomes (2L, 2R, 3L, 3R, X, 4) diagrammed in two-strand stage at the time recombination is effected, centromeres to left. Approximate positions of breaks indicated by gaps, the chromosome type occurring at the same level in both strands, as some of the breaks in rearrangements A to E, the chromatid type represented in only one strand. Portions of chromosomes identified in salivary gland nuclei represented in solid black; portions not recovered indicated by cross hatching. Rearrangement shown in A appeared as a mosaic, some salivary gland nuclei showing a reversed repeat type of duplication produced by parts shown in solid black, other nuclei showing a deficiency produced by combination of the stippled portions. When chromosomes other than those showing the duplicated sections were involved in a complex rearrangement, the positions of breaks in these chromosomes are also indicated. Thus, in H, duplicated sections of 2L were identified, but the rearrangement also involved breaks in 2R, 3R and Y. Rearrangement D had 14 breaks, although only 12 are shown for the reason that the positions of the breaks in one of two reversed repeats could not be identified.

random along the chromosomes as are the chromatid breaks. In terms of the 20 divisions into which each limb of a salivary gland chromosome of *D. melanogaster* has been divided, they were found in the third (near the tip), the fifth, sixth, eleventh, twelfth, eighteenth and twentieth (most proximal). Chromosome and chromatid breaks commonly occur

in the same limb. One rearrangement of special significance included two chromosome breaks, one proximal and the other distal, and in the region between them two chromatid breaks, one in each of the two strands. On the assumption that chromatid and chromosome breaks reveal whether a chromosome has or has not been divided, these cases indicate that the split has been only partially effected. It thus becomes necessary to assume that the chromosome is in a state of active or arrested division while in the sperm nucleus, and that its reduplication is not initiated in some specific region from which the split proceeds zipper-like either proximally or distally (cf. Darlington, 1937, p. 426). Since the condensed, inactive condition of the chromosome makes division at this stage highly improbable, there remain the possibilities that the chromosomes are either single or that they are longitudinally double throughout their lengths.

The former of these alternatives (recently stressed by Muller, 1940) would demand the production of breaks or potential breaks and their repetition in the second chromatid produced subsequently. Restitution must then occur, at least within the limits of the duplicated section, in one of the two strands at those levels in which a break is detectable only in the sister chromatid. Under such conditions a higher proportion of duplications might be expected than are detected, since breaks in two strands would be available for recombination. The break distribution that leads to inversion, for example, might conceivably produce a considerable number of viable reverse repeats and deficiencies if combination were possible among all eight of the broken ends available in two identical chromatids. Production of these two types of changes as reciprocal classes is shown by their discovery as mosaic tissue in one pair of salivary glands (fig. 1).

The possibility that each chromosome in the mature sperm may consist of two chromatids is supported by the considerable number of cytological studies of meiosis in organisms whose cells and chromosomes are larger and therefore more amenable to cytological analysis than are those of *Drosophila*. This interpretation of chromosome structure finds greatest support in the evidence presented by Sax (1938, also this volume) and others that both strands of a divided chromosome may be split simultaneously by a single "ionization." Demerec and Sutton (1940) have reported a Notch deficiency in *D. melanogaster* that is of some interest in this connection. In one chromatid obtained from the irradiated sperm there was a deficiency from band 3C8 to band 3E5 inclusive; in a sister chromatid a piece one band longer, extending from 3C7 to 3E5, had been removed and inserted in another chromosome. Demerec and Sutton are of the opinion that the probability is very small that two independent "hits" would occur in sister strands within the width represented by a single salivary gland chromosome band. Since other evidence which

they have collected indicates that the effect of a single "ionization" may spread along the chromosome, it seems probable that this change at the Notch locus furnishes an example of a similar effect which has traversed the space between chromatids. In the compact sperm head this distance may be as small as 150 Å. The evidence from chromatid and chromosome breaks presented in the present paper can likewise be interpreted best on the assumption that two adjacent sister chromatids may be affected at the same level by a single ionization.

Break Frequency-Dosage Relationships

Studies of break frequency at different dosage levels have been undertaken as part of the attack on the problem of the origin of structural changes. If individual "ionizations" can lead to disruption of intra-chromosomal connections, single breaks should increase proportionally with the dosage. But since such changes cannot be detected following irradiation of the sperm of *Drosophila*, analysis must be made of rearrangements which involve at least two breaks. If in their production breakage and recombination were part of a single process and occurred simultaneously, being dependent on propinquity or intimate contact of chromosomal regions, it would be expected that the frequency of rearrangements would increase proportionally with the dosage. If, on the contrary, "ionization" induces changes wholly independent of chromosomal connections, the detectable aberrations, each of which involves a minimum of two such changes, should increase in frequency at the lower dosage levels not less than in proportion to the square of the dosage. Although at this time the argument of delayed attachment can be mustered in discriminating between the first of these alternatives, the contact theory of Serebrovsky and the second or breakage theory of Stadler, it should be pointed out that the studies reviewed in the earlier part of this paper were outgrowths of experiments designed primarily to determine dosage relationships. At the time that the cytological analysis of salivary glands was first undertaken to measure quantitative relationships, the evidence previously obtained by genetic tests was in confusing disagreement, since the frequency of chromosomal rearrangements had been reported in different experiments to be either proportional or disproportional to dosage.

The first studies utilizing salivary gland chromosomes likewise were interpreted differently. Catchside (1938) regarded his data as favoring the concept of linear proportionality and the concomitant contact hypothesis. In the experiments of Bauer, Demerec and Kaufmann (1938) the frequency of altered sperms showed "a significant deviation from a linear proportionality." Subsequent analysis of these data indicated that the relationship of dosage to break frequency was represented by a curve intermediate between a straight line and a parabola. The slope of the curve was elevated

sharply at the 3000 roentgen level, but it is now clear that this departure was due to the inclusion of a group of slides from a mating of Swedish b by Oregon R flies which deviated markedly from the Oregon R by Oregon R material forming the larger part of the data.

Another factor apparent in the latter study which seemed to minimize the possibility of an underlying contact mechanism was the discovery of rearrangements incorporating as many as seven breaks. If contact were a prerequisite it would require a knot configuration similar to that suggested by Dubinin and Khvostova (1935) within which all strands could be sensitized by the effects of a single ionization.

The type of curve secured in the study by Bauer, Demerec and Kaufmann suggested that some general principle applicable to the dosage-break frequency relationship was being revealed, since similar curves have been obtained by Muller (1938) from genetic tests on *Drosophila* and by Sax (1938) from cytological studies on *Tradescantia*. Sax (1939) soon showed, however, that his results were attributable to a certain amount of restitution during the period of the treatment and that a second power curve could be approached if all the treatments of a dosage series were delivered synchronously by varying the intensity. It has already been shown that similar restitution does not occur in the sperms of *Drosophila*. Muller (1940) now credits the type of curve which he previously obtained and which he since has duplicated to the entrance of a "saturation effect" plus a disproportionately increased chance for inviable products among the multiple combinations.

Is then this intermediate type of curve the closest possible approach experimentally to the theoretically expected values? Bauer (1939) has extended the earlier data secured from Oregon R matings in a more comprehensive analysis. He has thus been able to confirm the interpretation that within the 1000 to 5000 roentgen range of dosage tested, the frequencies of altered sperms do not increase linearly with the dosage. More striking and significant is the close correspondence to the two power curve which has been shown to exist for total break frequency and for the percentages of independent rearrangements.

These findings in themselves indicate that breakage precedes recombination. But apart from this generalization, Dr. Ugo Fano has found by examination of these and additional similar data such inconsistencies at different dosage levels that the whole problem needs more careful consideration. This he has presented elsewhere in this volume.

The Effects of Neutrons

In inducing chromosomal derangements, X-ray treatments given in this laboratory utilized a Coolidge tube, operating at 5 ma. and at about 80 to 90 KV. so as to deliver rays of the order of magni-

tude of 0.2 Å. Muller (1940) has reported that there is no difference in frequency of translocations attributable to such different wave lengths as are produced by 50 KV. X-rays and γ -rays of radium. Independence of wave length within the range from soft X-rays to γ -rays had previously been found in measurements of the production of recessive lethals in *Drosophila* (see for example review by Timofeeff-Ressovsky, 1937; Fricke and Demerec, 1937), and led to the conclusion that localized effects were due to single "ionizations" rather than the absorption of quanta. Timofeeff-Ressovsky and Zimmer (1938) found, however, that fast neutrons gave lower mutation rates than the equivalent doses of X-rays. Since these results were at variance with others which indicated a greater effectiveness of neutrons (cf. Nagai and Locher, 1938, on *Drosophila*) it was decided to make further measurements of mutation rates as well as the capacity of fast neutrons to produce chromosomal breaks.

Two treatments of Oregon R males of *D. melanogaster* were given by Dr. M. A. Tuve of the Department of Terrestrial Magnetism of the Carnegie Institution of Washington with the aid of the Institution's electrostatic generator. Dosages were measured with a Victoreen dosimeter and estimated by Dr. Tuve to be the equivalent of 3000 and 4000 roentgens of X-rays. Genetic tests were conducted by Dr. M. Demerec; slide analysis was made by Dr. Eileen Sutton and the writer.

The frequency of sex-linked lethals at the lower dosage was 1.88 ± 0.39 percent and at the higher dosage 2.81 ± 0.51 percent. Control values for lethals produced by 3000 and 4000 roentgens of X-rays are 2.15 ± 0.67 and 3.08 ± 0.69 respectively. Neutrons are also effective in producing chromosomal rearrangements. At the lower of the two dosages the percentage of altered sperms was 18.43 ± 2.43 and the percentage of breaks per total sperm 46.66. Corresponding values for the equivalent 3000 r X-ray treatment are quite similar, namely, 18.82 ± 1.60 and 49.23 percent. The higher neutron dosage gave, however, only 20.2 ± 2.33 percent of altered sperms and 53.5 percent of breaks as compared with the 30 and 86 percent obtained with the equivalent 4000 r of X-rays. The more gradual rise in frequency of aberrations with fast neutrons than with X-rays has also been reported by Giles (1940) who found in *Tradescantia* a relationship that appeared to be proportional to the dosage. The types of rearrangements induced by neutrons in *Drosophila* sperms are similar to those produced by X-rays, with the possible exception that neutrons may be more efficient in inducing intercalary deficiencies. Among the 107 altered sperms analyzed, 12 deficiencies were found. Bauer (1938) reported only 13 among 688 sperms showing X-ray effects. Distribution among the chromosomes of breaks induced by neutrons seems to be essentially at random insofar as can be determined by the rather small sample of 233 observed.

Differences between the effects of X-rays and fast neutrons have been attributed to the denser "ionization" occurring along the path of the recoil protons than along the electron path. A widespread effect along a proton track may possibly account for the considerable number of deficiencies produced in our material by the neutrons. It is probably not effective in causing simultaneous disruption of the two or more loci entering into a chromosomal rearrangement, as Giles believes may happen in *Tradescantia*, for the reasons that break distribution, and types and frequencies of rearrangements in *Drosophila* are so nearly comparable with those produced by X-rays. The more plausible conclusion to be derived from the limited data here presented, which unfortunately cannot be extended at present, is comparable with that drawn by Timofeeff-Ressovsky and Zimmer, namely, that a saturation effect enters at a much lower dosage level than with X-rays, so that a considerable portion of the ionization is superfluous within the sensitive volume and therefore is ineffectual in producing structural changes. The data here presented contrast with those of Nagai and Locher and of Giles, and are in harmony with those of Timofeeff-Ressovsky and Zimmer in showing that comparable doses of neutrons do not produce higher frequencies of detectable changes than X-rays.

SUMMARY

Salivary gland chromosomes of the Diptera provide unique and unparallelled material for quantitative studies of the effects of irradiation. In *Drosophila* such analyses have shown that breaks are distributed at random along the chromosomes except for the proximal heterochromatic regions and for similar intercalary regions. The data are in harmony with the theory that breaks in a given region occur in proportion to the length represented by that region during late prophase or metaphase of mitosis.

Changes induced in the chromosomes of the sperm are not effective at once in producing rearrangements and probably not until the male pronucleus is formed within the egg. Several lines of evidence indicate that these changes represent potential breaks. The suggestion is offered that they may only be effective if combination with other similar regional alterations is realized.

Recombination is not entirely at random but is apparently favored by propinquity of the potential breaks, so that when only a few breaks are involved it appears that a restricted recombination zone exists.

Evidence from distribution of chromosome and chromatid breaks indicates that the chromosomes of the mature sperm consist of two separate chromatids, both of which can be severed at the same level by the effects of a single "ionization."

The number of altered sperms does not increase linearly with the dosage. The frequency of breaks and the frequency of independent rearrangements

produced at different dosage levels indicate that the increase follows more nearly the square of the dosage in the range between 1000 and 5000 roentgens.

All of these findings support the concept that breakage precedes recombination rather than the concept that breakage and recombination are part of a single process and occur simultaneously.

Neutrons give a similar or somewhat lower, but not a higher break frequency than X-rays of comparable dosage. These results may be due to a saturation effect being reached at lower doses with neutrons than with X-rays.

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DISCUSSION

MULLER: In your tables, what is the meaning of the value for "distance between breakage points" in the case of reciprocal translocations?

KAUFMANN: Distance of the breaking points from the centromeres was determined for the two arms involved; the value given in the table shown represents the difference between these two measurements.

MULLER: Does the distribution of break points in inversions considered by themselves agree with the expectancy?

KAUFMANN: The agreement is close, about .6 to .7 when measured by the Chi square test.

MULLER: Sidky's case is not explicable as having occurred wholly in a nucleus of paternal origin, because this would have required a Y to have been present in the paternal gamete as well as in the maternal, whereas no extra Y was present in the individual which contained the translocation.

KAUFMANN: Under those conditions it must be assumed that a spontaneous break has occurred in the Y chromosome.

SCHULTZ: What is the present status of the experiment in which irradiation of males at low temperature was carried out and an increased frequency of translocations detected by genetic means? The different experiments give results in the same direction, a slight increase of translocations at the low temperatures.

MULLER: The results of this kind which I reported in 1930 were not reliable, as I stated at that time; results which we have obtained since then show no such effect of temperature.

KAUFMANN: No conclusion is possible concerning infrared. The experiments have just begun.

GLASS: Further evidence from *Drosophila* supports the conclusion of Kaufmann that propinquity of breakage points favors recombination. From X-ray treatment of oocytes and oogonia, I have been unable to obtain translocations at all, although some inversions appear. From the data of other workers, it seems that the frequency of inversions is possibly not much below that of X-rayed sperm.

KAUFMANN: Would you attribute this propinquity to some coiling mechanism within the chromosome?

GLASS: I am not prepared to give a definite interpretation at this time.

METZ: What is meant by support of the breakage first hypothesis and not the contact hypothesis?

KAUFMANN: The contact hypothesis of Serebrovsky implies that breakage and recombination are part of a single process and occur simultaneously. The data presented here do not support this interpretation.

HOLLAENDER: The question has been raised as to whether the effects of infrared are not really heating effects. Localized heating may occur but not heating of the whole fly above 27°, because we have taken precautions to cool the vials, etc.

SCHRADER: What connects the parts bordering such a secondary constriction as you have shown to exist in the second chromosome—a chromatic thread?

KAUFMANN: I assume from the appearance when the regions bordering the constriction are close together that they are connected by a chromonematic thread. This is not the primary constriction, but a secondary one, not, as far as I have seen, concerned with nucleolus organization.

SCHRADER: One would think that if this part is thus extended at the time of X-raying, it would show more breaks than other regions of the chromosome.

KAUFMANN: Although this constriction borders the heterochromatin, a comparison is difficult because we are raying mature sperm where the structure of the chromosome cannot be determined.

DELBRÜCK: I want to stress the fact that a hit does not produce a break proper, but the events which follow it do so.

Kaufmann says that his data favor the two-strand stage in the sperm nucleus; is this based on chromatid breaks?

KAUFMANN: On distribution of chromatid and chromosome breaks in my material and on the case of Demerec and Sutton referred to in this paper.

DELBRÜCK: If the sperm has single strands, then we may assume as an alternative explanation of your results that there are potential breaks of two kinds, one resulting in breaks of both daughter strands, the other of only one daughter strand.

KAUFMANN: We must accept this.

MULLER: If the chromosome is single in the sperm, there must be a delay of union in some cases until after the chromosome divides, and a broken end need not have found another broken end to unite with before its division. It seems from Kaufmann's and other evidence that unions more usually come before division but not always.

SAX: Evidence from *Tradescantia* shows that breaks and fusions are not delayed for very long periods, since cells irradiated at the resting stage show only the normal chromatid aberration frequency when subsequently rayed at prophase.

STADLER: The question of the potential break is borne upon by the effects of ultraviolet and X-rays.

CHILD: The infrared work bears upon the question of potential breaks, since many photochemical reactions are reversed by infrared. The ultraviolet or X-rays may produce the latent change in the gene or chromosome, which change goes on to completion after the direct radiation. The infrared, in reversing the reaction before its completion, restores the original state. I therefore favor the concept of latent breaks.

NEBEL: The work of Charles and Nebel favor this idea and I support this theory.

HUSKINS: Direct evidence from Trillium (1935) showed: a) that the break does not occur immediately, and b) that only one of the chromatids may be broken.

KAUFMANN: In *Drosophila*, we must account for breaks or potential breaks persisting for periods up to 30 or more days.

METZ: In *Sciara*, no potential breaks are obtained from eggs rayed before meiotic movements begin; if later, breaks are recovered.

STADLER: I do not think we can determine from the present evidence what is occurring in the interval between the treatment and the realization of the derangement which is later observed. The evidence of "breakage first" shows merely that some change is produced at separate points in the gene string; and that later there are interchanges at these points. There is no evidence that the fragments which will be translocated are free during the interval. This evidence may be provided by the tissue culture experiments which Carlson is starting.

METZ: Reynolds, working on *Sciara* eggs, found that metaphase and anaphase stages give rearrangements. The whole process is completed before this meiotic division is completed.

TYPES AND FREQUENCIES OF CHROMOSOMAL ABERRATIONS INDUCED BY X-RAYS

KARL SAX

Previous analyses of X-ray induced chromosomal aberrations in *Tradescantia* microspores have led to the following conclusions. Either one or both of the sister chromatids at prophase can be broken by a single "hit." Exchanges or fusions between chromatids of different chromosomes are the result of two independent "hits," as are the ring and dicentric chromosomes induced during the resting stage. When the time of irradiation is constant for different X-ray doses the aberration frequency increases as the square of the dosage, but when X-ray intensity is constant the exponent of the dosage curve equation is less than two. The effectiveness of the time-intensity factor suggests that both breakage and fusion may occur during the time of exposure. Fractional dosage experiments have shown that most breaks undergo either restitution or produce chromosome aberrations within about an hour after irradiation (Sax, 1938, 1940).

The effect of the time-intensity factor has been confirmed in *Tradescantia* by Fabergé (1940), but it is not effective in reducing aberrations in *Drosophila* sperm (Muller, 1940; Kaufmann, 1941). In *Drosophila* the broken ends of chromosomes in the sperm do not fuse until fertilization. The relation between dosage and aberration frequency is reported as linear in *Tradescantia* by Fabergé and in *Vicia* by Marshak (1939), and in *Drosophila* Muller found an exponent of 1.5. This exponent does not necessarily mean that aberration frequency fails to increase as the square of the dosage, since the analysis was made on the aberrations surviving a number of cell generations. The production of two breaks by a single "hit" has been questioned by Delbrück (1939) on purely physical grounds, and the breaks in both sister chromatids at the same locus have been attributed to chromosome division before the fusion of broken ends in the single thread (Muller, 1940; Darlington and Upcott, 1941). However, the production of breaks in both chromatids by a single hit is supported by the work of Nebel (1937) in *Tradescantia*, by Carlson's (1938) analysis of *Chortophaga* chromosomes, and by a number of experiments with *Drosophila* (Kaufmann, 1939; Kaufmann and Bate, 1938; Demerec and Sutton, 1940).

The following experiments were designed to determine the relation between X-ray dosage and aberration frequency at different intensities, the effect of fractional dosage, and the nature of the breaks induced in sister chromatids.

EXPERIMENTAL METHODS

Microspores of *Tradescantia paludosa* were used

for most of the experiments. The X-ray tube was calibrated by Dr. Hudson and most of the subsequent exposures were measured with a Victoreen dosimeter provided by Dr. Curtis of the cyclotron laboratory. Measurements made simultaneously with the two dosimeters were identical. The maximum intensity used was 160 r/m at 26 cm. from the target. Closer distances cannot be used with uniform results for *Tradescantia* buds. It was found that the output of the tube had decreased about ten percent since its calibration for earlier experiments three years ago. The tube was always warmed up for about five minutes before an exposure to avoid fluctuation in amperage. The tube was operated at 10 ma. at a peak potential of 160 KV. The intensity was varied by changing the distance between the target and the inflorescences for most experiments, but for doses at 2.7 r/m the amperage was reduced to 8 ma. and a 0.7 mm. aluminum screen was placed over the buds. The elimination of the longer wave lengths is of no consequence since it has been shown that aberration frequency is independent of wave length in the normal X-ray range (Fabergé, 1940; Muller, 1940; Rick, unpublished).

It was found that X-ray doses given at 40 r/m or less could be calibrated with a fair degree of accuracy by time of exposure alone. Most of the irradiation without dosimeter measurements was done at 40 r/m.

In earlier experiments it was found that a sample of division figures from three slides gave fairly consistent results. According to Fabergé, thirty division figures from each slide are adequate, and for high aberration frequencies fewer cells can be used. Variability is greater between slides than within slides, so that the number of buds sampled should be used as the unit for statistical treatment. Where the aberration frequency is low it is not possible to obtain a sufficient number of division figures from a single bud for an adequate sample. If the aberration frequency is to be accurate within 10 percent, the number of chromosomes necessary for analysis is $n p / \sqrt{n p q} = 10/1$ where n is the number of chromosomes which must be examined, p the probability of a chromosome break, and q the probability of no break. The values of n for different percentages of breaks are shown in Figure A. For a 1 percent break frequency nearly 10,000 chromosomes should be analysed to obtain an accuracy within 10 percent of the mean and not until the frequency of breaks is between 20 and 30 percent is it possible to obtain accurate results with an analysis of only several hundred chromosomes. The larger chromosome numbers necessary for the determination of the

smaller values can be obtained only by combining the data from different slides, and then increased variability is introduced because of the variation between buds even in a clonal line. The total number of buds available for replications is limited because of technical difficulties in preparing sufficient

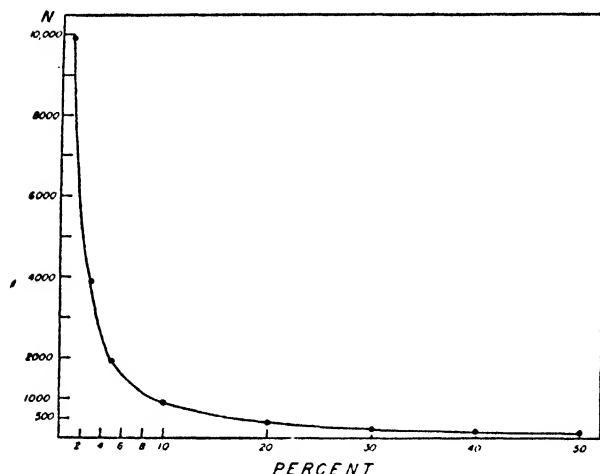


FIG. A. The value of n necessary for obtaining a mean value accurate within 10 percent is shown for various means from 1 to 50 percent. This graph was prepared by Dr. Sheldon Reed.

slides for a complete dosage experiment within critical time limits. Material for chromatid aberrations must be fixed within a period of about an hour for comparable results, owing to differential sensitivity during the nuclear cycle. We have usually analysed one hundred division figures from each of six slides. The probable errors are based on the variation between slides.

The analysis of chromatid and chromosome aberrations is quite simple, particularly at the lower frequencies. Miss Margery Poole has done most of this work, although I have often analysed several slides in each series of a dosage experiment and complete series of several experiments. Our results are in close agreement, and agree with the results of Dr. Charles Rick and Dr. Norman Giles who have worked on similar material. Dr. Nebel and I have exchanged slides for analysis and our results also are quite similar.

The frequency of spontaneous chromosomal aberrations in normal diploid *Tradescantia microspores* is only 0.04 percent and they are almost exclusively one-hit chromatid aberrations (Giles, 1940).

In calculating the percent of breaks, a simple deletion is classed as one break while aberrations involving two different chromosomes or different loci of the same chromosome are classed as two breaks.

TYPES OF ABERRATIONS

With low dosage the physiological effect of X-rays is limited so that true chromatid aberrations can be

observed within three hours after irradiation. Only chromatid aberrations are found during the first 25 hours after raying. These chromatid alterations are of two general types, those resulting from a break at a given locus of one or both of the sister chromatids, and those resulting from two independent breaks in the chromatids of different chromosomes or at different loci of the same chromosome. The one-hit aberrations may involve only one of the two chromatids (Plate I, fig. 1), or both sister chromatids may be broken followed by lateral fusion to produce a dicentric chromatid and an acentric U-shaped fragment (Plate I, fig. 1). Only in very rare cases do the ends of two broken sister chromatids fail to fuse. At metaphase the single deletion usually is associated with its sister chromatid and often is included among the centric chromosomes at anaphase (Plate I, fig. 2). The double deletion becomes straightened out and usually is left free in the cytoplasm at anaphase (Plate I, fig. 3).

The two-hit chromatid aberrations include reciprocal interchanges or fusions between chromatids of different chromosomes (Plate I, fig. 4). The fusions, which are about twice as frequent as the interchanges, are accompanied by acentric fragments. Ring chromatids accompanied by acentric fragments are found occasionally (Plate I, fig. 5). Another type of aberration is shown in Plate I, Figure 6. One of the chromatids is buckled as though there had been an intercalary duplication and deficiency, but often the ends of the protruding buckle do not appear to be fused. Similar types of aberrations are occasionally found also among chromosome breaks induced during the resting stage, and these may be lateral translocations (cf. Sax and Mather, 1939).

During the transition from chromatid to chromosome breaks when both types are present, the double deletions or the broken ends of the centric chromatids may fail to fuse (Plate I, figs. 6 and 7). In rare cases fusion may be incomplete as indicated by an achromatic lesion at the point of union (Plate I, fig. 7), but in nearly all fusions the union is so complete that its locus cannot be detected. Broken ends of centric chromatids which fail to fuse appear to behave like normal ends of chromosomes, although the subsequent division in the pollen tube does occasionally include a dicentric chromatid with no fragment. It is known, however, that a broken end of a chromosome can behave like a normal end in *Zea* (McClintock, 1941), and in *Drosophila* (Sutton, 1940).

Chromosome breaks induced in the resting stage, before the chromosomes are effectively split, produce chromosome aberrations of three general types. Terminal deletions are produced by single hits. The broken ends of chromatids fail to fuse at anaphase so that the centric chromatids separate freely and the acentric fragment consists of two chromatid segments. Such deletions constitute only about 5 percent of all chromosome aberrations. The two-hit

aberrations include ring and dicentric chromosomes accompanied by acentric fragments (Plate I, figs. 8 and 9), interstitial deletions (Plate I, fig. 11), inversions and translocations (Plate I, fig. 12). Some of the interstitial deletions are produced by single hits. Since Rick (1940) has analysed the terminal and interstitial deletions in considerable detail, they have not been included in the following analyses. Inversions can be recognized only when the centromere is shifted considerably, and translocations can be detected only when they are unequal, so both of these classes of aberrations have not been included. Analyses of chromosome aberrations are based only on rings and dicentrics, both of which can easily be detected.

FREQUENCIES OF TYPES OF CHROMATID ABERRATIONS

The occurrence of only chromatid aberrations during the first 25 hours after irradiation indicates that the chromosomes must have been effectively

TABLE 1. PROPORTIONS OF TYPES OF CHROMATID ABERRATIONS INDUCED DURING PROPHASE DEVELOPMENT 120r. Sept.

Types	Time after raying			
	6 hrs.	12 hrs.	24 hrs.	30 hrs.*
Single deletions	34%	30%	23%	8%
Double deletions	27%	44%	56%	70%
Exchange	39%	26%	20%	22%
No. of aberrations	202	561	343	86

* Both chromatid and chromosome aberrations present.

split into sister chromatids when rayed. At earliest prophase, just after the formation of sister chromatids, the two threads are closely associated, but as prophase develops the chromatids gradually become more separated as minor coiling develops. Since the range of an X-ray hit must be quite restricted the proportions of single and double deletions should vary during the prophase cycle. At late prophase when the sister chromatids are clearly distinct one would expect more aberrations involving only one of the sister chromatids than those involving breaks in both sister chromatids. The results of such an analysis are shown in Table 1. In material fixed at 6, 12, 24, and 30 hours after raying there is a gradual decrease of single deletions and chromatid fusions or exchanges as the time of fixation proceeds towards earliest prophase.

In cells rayed at late prophase double deletions are comparatively infrequent, but with irradiation at early prophase when the chromatids are close together the frequency of double deletions is greatly increased. The transition in all classes is a gradual one. The results of this experiment, and the fact that the frequency of double deletions increases directly with X-ray dosage, proves conclusively that

a single "hit" can break both sister chromatids even when they are far enough apart to be differentiated easily in fixed preparations under moderate optical magnification. The excess of single deletions, and of exchanges or fusions between chromatids of different chromosomes at late prophase, indicates that at this stage a single hit more often breaks only one of the two sister chromatids.

TRANSITION FROM CHROMOSOME TO CHROMATID ABERRATIONS

Several investigators have suggested that the presence of chromatid aberrations is no indication that the chromosome was split into sister chromatids when irradiated. If restitution or illegitimate fusion of broken ends is delayed, a chromosome rayed in the resting stage could become effectively split and fusions could occur between chromatids to produce the typical chromatid aberrations. Such a condition might be possible in *Drosophila* sperm where the broken ends do not fuse until fertilization of the egg, long after the breaks were induced. In *Tradescantia*, however, the time-intensity and fractional dosage experiments indicate that restitution or illegitimate fusions occur soon after the breaks are induced and that practically all fusions are effected within an hour after raying. Under such circumstances we should expect that the transition from chromosome to chromatid breaks should be effected in a comparatively short time.

During the winter months when the nuclear cycle, —from meiosis to the division of the microspore nucleus, is about ten days, the transition from chromatid to chromosome aberrations occurs at about two days after raying (table 2). At 48 hours the chromatid aberrations, and especially the two-

TABLE 2. TIME OF TRANSITION FROM CHROMATID TO CHROMOSOME BREAKS Jan. 17. 160r.

Time of fixation	Total Chromosomes	Chromatid		Chromosome Dic. & Ring
		1-hit	2-hit	
24 hr.	8724	5.2%	5.5%	0
48 hr.	6174	3.8%	0.9%	1.8%
6 day	8868	0		3.7%

hit aberrations, decline in frequency as the chromosome aberrations appear.

A more complete analysis of the transition period was made in April and May when the duration of the nuclear cycle is about a week (table 3). Only chromatid aberrations were observed at 23 and 25 hours after raying, and at 26 hours the first chromosome aberration was found. Between 25 and 31 hours the frequency of chromatid aberrations decreased as the frequency of chromosome aberrations increased. The one-hit chromatid aberrations persist somewhat longer than the two-hit chromatid

aberrations. Most of the transition from chromatid to chromosome breaks occurs between 27 and 31 hours after raying. The maximum frequency of chromatid aberrations, however, is at 20 to 25 hours after raying (cf. Sax and Swanson, 1941). The de-

TABLE 3. TRANSITION FROM CHROMATID TO CHROMOSOME BREAKS
160r. Apr.-May. S.B.S. Clone. Two series

Hrs. after raying	Total Chromo- somes	Chromatid		Chromo- some R.+D.
		1-hit	2-hit	
		%	%	%
23	372	6.7	5.9	0
25	828	5.3	3.6	0
26	372	5.1	2.2	0
27	936	3.8	1.7	1.3
28	600	2.3	0.7	3.3
29	1152	3.4	1.1	1.7
30	1014	2.8	1.4	2.7
31	600	3.2	1.3	3.7
32	960	0.8	0.0	3.3
33	990	2.2	0.2	3.3
34	600	0.8	0.0	4.0

crease in the frequencies of two-hit chromatid aberrations during the transition period is much more striking than the decrease in one-hit aberrations.

A transition period of about four hours suggests that there is considerable variation in time of chromosome splitting or that some of the chromosome breaks fail to fuse until the chromatids are differentiated. The rare occurrence of both chromosome and chromatid aberrations in the same chromosome suggests that the effective splitting must occur rapidly or that there is considerable variation in the time of fusion following breakage. It is possible that many of the chromatid aberrations found

during the transition period from 27 to 31 hours after raying are produced by breaks in the single thread stage which do not fuse until the chromosome is effectively split.

It is possible, however, that for a short time after effective splitting of the chromosome breaks involving both chromatids can give rise to either chromatid or chromosome aberrations. The maximum frequency of chromatid aberrations occurs several hours before any chromosome aberrations appear. These, as well as all earlier aberrations, must be derived from breaks originally induced in the differentiated chromatids.

DOSAGE CURVES

The determination of the relation between dosage and aberration frequency is of fundamental importance in the interpretation of the nature of X-ray action in producing breaks in chromosomes. If an aberration is produced by a single hit the relation between dosage and aberration frequency should be linear, while if two hits are necessary the frequency should increase as the square of the dosage. The analysis is complicated due to the fact that both breaks and fusions occur during the time of exposure. Thus the relation between dosage and frequency of two-hit aberrations should vary with X-ray intensity.

A repeated analysis of one-hit and two-hit chromatid breaks confirms earlier observations. The data are shown in Table 4. It is evident that the one-hit aberrations,—in this case the double deletions, increase in direct proportion to X-ray dosage. The two-hit aberrations increase as the square of the dosage.

The relation between dosage and chromosome

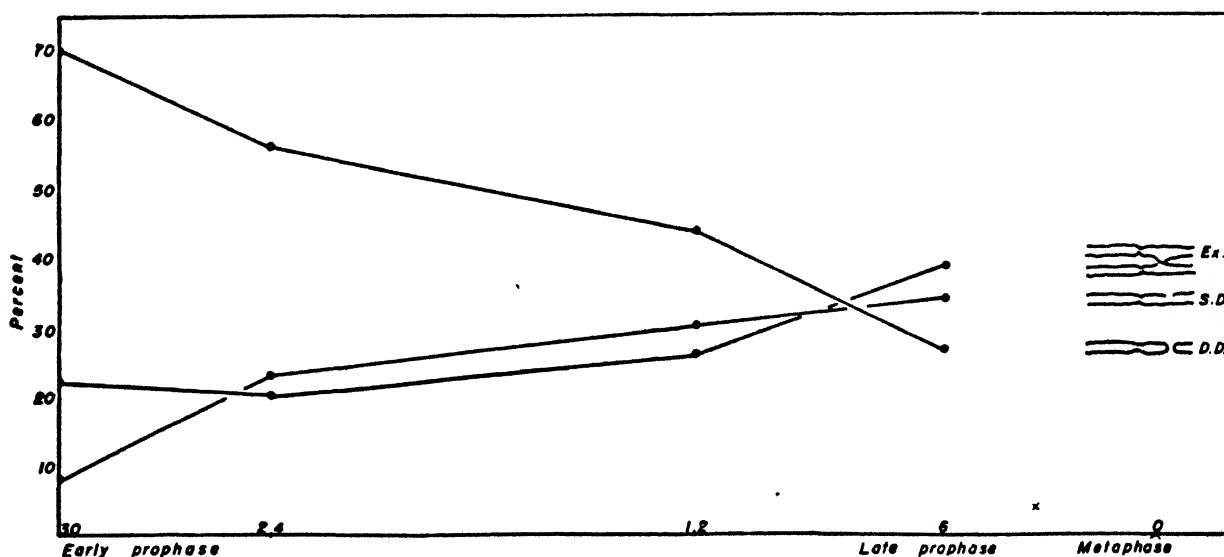


FIG. B. Variation in proportions of types of chromatid aberrations produced by raying the microscore nucleus at various stages of prophase development. Data from table 1.

aberrations has been analysed in considerable detail. Only dicentric chromosomes and centric ring chromosomes have been included in the analyses since these aberrations are easily observed, and it is clear that they are derived from breaks in different chromosomes or breaks in the two arms of the same

TABLE 4. DOSAGE CURVE FOR CHROMATID ABERRATIONS
40 r/m. Fixed at 24 hrs.

Dose in r	Chromatid aberrations		Total Chr.
	1-hit	2-hit	
10	1.3	0.5	1326
80	2.2	2.7	1200
160	5.2	10.4	1518

chromosome. In the first analysis the dosage was increased by increasing the time of exposure and the X-ray intensity was 25 r/m. Under these conditions a dosage curve was derived which had an exponent of 1.5 (Sax, 1938). In later experiments the dosage was varied by varying the distance between the target and the flower buds, according to the inverse square law. The dosage range was from 100 r to 500 r, and the maximum intensity was 200 r/m. The exposure time was 2.5 minutes for all doses. The entire series of exposures were made simultaneously so that the relative doses were proportional even though the absolute dosage may not have been exact. Under these conditions the aberration frequency increased as the square of the dosage (Sax, 1940).

TABLE 5. DOSAGE CURVE
Time constant. 160 r/m

Dose in r.	Total		Percent Breaks	Theor. (D/82) ²
	Slides	Chrom.		
120	6	3600	2.6 ± 0.3	2.1
240	6	3300	8.4 ± 0.4	8.5
360	6	900	18.4 ± 1.9	19.3
480	6	900	34.0 ± 1.4	34.2

This experiment was repeated after the X-ray tube had been carefully recalibrated with a dosimeter. All exposures were made simultaneously in a three minute time interval. The results of this experiment are shown in Table 5. The aberration frequency increased as the square of the dosage. The dosage curve plotted from the derived equation is shown in Figure C.

Since the aberration frequency is related to the time-intensity factor a series of experiments were devised to test the effect of various intensities of radiation. The dosage was varied by increasing the time of exposure. Each exposure was measured with a dosimeter. The maximum dosage which could be recorded on the dosimeter was 250 r, so that the

higher doses had to be fractionated to permit dosimeter readings. The time necessary to read the dosimeter was of little consequence at the lower intensities, but at 160 r the total exposure time was doubled at the highest dosage. The maximum dosage was limited to 480 r because higher dosages at high intensity produce too many aberrations for accurate analysis. The second series was rayed at 20 r/m, and the third at 160 r/m. The results of these experiments are shown in Table 6, and in graphic form in Figure C. The probable errors were determined as $\chi_2\sigma$, where χ_2 is a constant for a given value of n , obtained from Pearson's tables. Dr. Sheldon Reed has calculated the standard error of estimate for the dosage curve obtained at 160 r/m. This value was found to be 0.8 percent and is indicated by a bar at the end of the curve shown in Figure C.

The basic dosage curve was obtained from the equation $(D/82)^{2.0}$ derived from the data shown

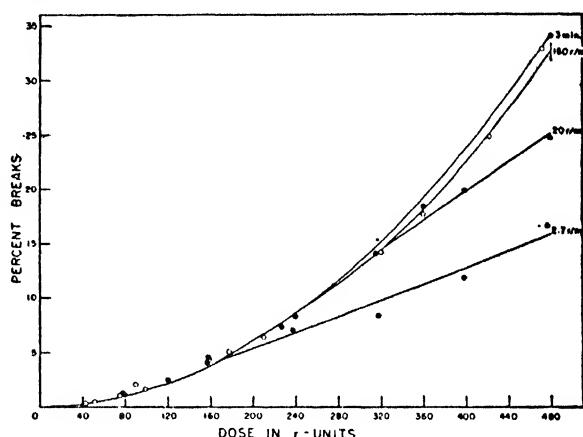


FIG. C. Dosage curves for two-hit chromosome aberrations at different X-ray intensities. The upper curve is based on data from a single three-minute exposure for all points. The dosage for the other curves was increased by increasing time of exposure at a given intensity.

in Table 5. The other curves are drawn only when they deviate from the basic curve. The values for exposures at 160, 20 and 2.7 r/m do not deviate significantly up to total doses of nearly 200 r, and are somewhat higher than those of the basic curve at doses from 80 to 160 r. The deviation of the 160 r/m curve deviates little from the basic curve at higher doses although it is slightly lower. The equation for this curve is $(D/75)^{1.85}$. The 20 r/m curve begins to deviate from the basic curve at about 300 r, and drops sharply at higher doses. No satisfactory equation can be derived for this curve since it is not uniform, but the aberration frequency increases approximately as the 1.5 power of the dosage. At 2.7 r/m the curve begins to deviate from the basic curve at about 200 r, and the aberration frequency at 480 r is only half as great as that obtained at 160 r/m. Although this curve is not uniform it is evident that it approaches linearity.

THE TIME-INTENSITY FACTOR

The decreased aberration frequency at lower radiation intensities would be expected if the time-intensity factor is operative. Since the frequency of breaks is based entirely on two-hit aberrations a decrease in aberration frequency at low intensities may be attributed to restitution of breaks during the time of exposure so that fewer adjacent breaks are

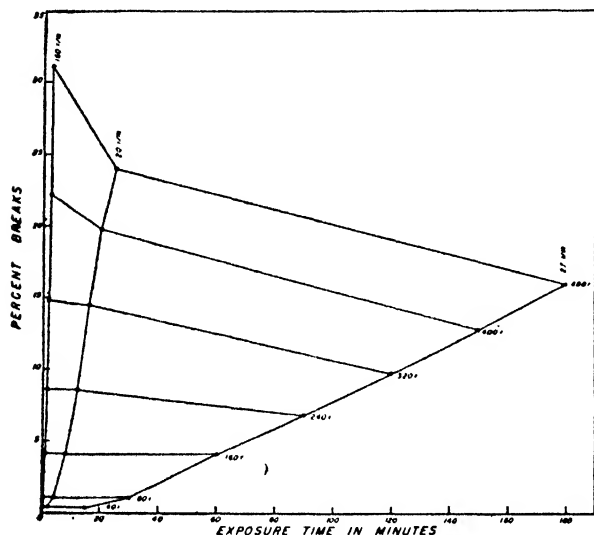


FIG. D. The relation between time-intensity and aberration frequency.

available for the production of dicentric and ring chromosomes. At high intensities, or at doses which are given in relatively short periods, all or most of the breaks remain open so that a maximum number of illegitimate fusions can occur. The data from Table 6 and Figure C have been used to show the relation between aberration frequency and exposure time at various doses and intensities of irradiation (fig. D).

It is evident that the time-intensity factor is most effective at the higher doses, which is in accord with Fabergé's (1940) results with similar material. At 480 r, a marked drop is found when the exposure time is increased from 6 to 24 minutes. This decline in aberration frequency becomes less at lower doses until at 160 r and less there is no evidence of any decrease in aberration frequency, even when the time of exposure is as long as an hour. The smaller values obtained at low doses are subject to considerable error due to inadequate samples (cf. Plate I, fig. 1) and natural variation in susceptibility of different inflorescences. However, there is clear evidence of a decreased effect of the time-intensity as the total dosage is decreased. The effectiveness of the time-intensity factor at the higher doses may be attributed to relatively rapid restitution of breaks so that as the time of exposure is increased there is less chance of producing two adjacent breaks within

the critical limits of time and space. The decline in the effect of the time-intensity factor at lower doses may be associated with a slower rate of restitution.

THE EFFECT OF FRACTIONAL DOSAGE

Fractional dosage provides a means of testing the validity of classification of the one-hit and two-hit aberrations, and permits the determination of the time during which breaks can remain open before restitution or illegitimate fusion. Previous experiments have shown that the X-ray induced breaks remain open for only about an hour in *Tradescantia*

(1) TABLE 6. DOSAGE CURVES
1 = 2.7r/m. 2 = 20r/m. 3 = 160r/m.

	Dose in r	Slides	Chromo- somes	Dicentric & Rings	Percent breaks
(1)	78	2	1200	8	1.3
	158	5	3066	65	4.6 ± 0.4
	237	9	1620	60	7.1 ± 0.3
	318	10	1968	87	8.4 ± 0.7
	398	7	1986	117	11.9 ± 0.5
	477	9	3054	246	16.7 ± 1.1
(2)	79	7	4200	26	1.2 ± 0.1
	157	6	1944	39	4.1 ± 0.3
	237	8	3040	110	7.4 ± 0.6
	315	6	1050	71	14.1 ± 1.8
	398	7	1638	159	19.9 ± 1.3
	479	9	1350	167	24.7 ± 1.9
(3)	43	5	4542	8	0.4 ± 0.04
	51	4	2190	6	0.5 ± 0.3
	75	4	4230	20	1.1 ± 0.1
	90	6	3600	38	2.1 ± 0.1
	99	4	2976	25	1.7 ± 0.3
	158	5	5400	117	4.4 ± 0.8
	177	7	4200	107	5.1 ± 0.5
	210	5	2826	89	6.4 ± 0.5
	320	5	2736	193	14.2 ± 0.4
	360	6	3600	319	17.7 ± 1.5
	422	6	3546	443	24.8 ± 0.7
	472	6	2586	419	32.8 ± 1.2

microspores (Sax, 1940), but in *Drosophila* sperm they may remain open for several weeks (Muller, 1940; Kaufmann, 1941).

The exposures were not calibrated in the following experiments, but at 40 r/m the exposure time alone gives values which are within 5 percent of dosimeter readings. The first experiment was done with chromatid breaks where adequate numbers of both one-hit and two-hit aberrations can be analysed. Two control exposures were made, one at four minutes to obtain the aberration frequency of continuous exposure, and one at one minute for the determination of the base line. If no restitution occurs during fractional dosage, the value obtained from the one-minute exposure multiplied by four should be the same as that for the single four-minute exposure. If restitution is complete during the rest periods between exposures, the fractional dosage should give values no higher than four times that

obtained from a one-minute exposure and both should be significantly lower than the aberration frequency from the continuous four-minute exposure. The results of this experiment are shown in Table 7. Fractional dosage with rest periods of 20, 40, and 80 minutes respectively does not decrease the frequency of one-hit aberrations. The curve for two-hit aberrations reaches the base line with 40-minute rest periods. The value for 20-minute rest periods is not very critical due to the small number of chromosomes available for analysis. The production of terminal deletions by single hits and the production of chromatid exchanges by two inde-

TABLE 7. EFFECT OF FRACTIONAL DOSAGE ON CHROMATID ABERRATIONS
40 r/m. Fixed at 24 hrs.

No. of exposures	Duration of exposures	Rest periods	Total Chr.	Percent breaks	
				1-hit	2-hit
1	4'	0	1482	4.6	9.9
4	1'	20'	648	3.7	9.9
4	1'	40'	1188	3.9	6.7
4	1'	80'	1260	4.0	5.1
1	1'	0	1770	1.1	1.6

$$B=4.4 \quad B=6.4$$

pendent hits is confirmed, or at least strongly supported, by these data. Chromatid breaks undergo restitution or illegitimate fusion within about 40 minutes.

The effects of fractional dosage on chromosome rings and dicentrics are shown in Table 8. Doses of 160 r at 40 r/m with rest periods from 20 to 240 minutes produce significantly lower aberration frequencies than the single four-minute exposure. Rest periods of 60 to 240 minutes result in values definitely higher than the base line, but the value for the determination of the base line is subject to considerable error. For doses of 320 r at 40 r/m the aberration frequencies of fractional doses reach the base line in an hour or less and do not rise significantly above the base line with rest periods of 120 or 240 minutes. At 320 r at 180 r/m the base line is reached with about 30 minute rest periods between exposures. The doses in this experiment were calibrated with a dosimeter and the time-intensity factor used in the table is accurate within ± 2 percent. In general the time necessary for complete restitution of broken ends of chromosomes is less than an hour and may be less than 20 minutes.

CONCLUSIONS

The data derived from dosage curves and fractional dosage experiments support the assumption that terminal deletions of chromosomes are caused by single hits. The dosage curves for both chromatid and chromosome (Rick, 1940) terminal deletions are approximately linear. Fractional dosage

has little or no effect on the frequencies of chromatid terminal deletions, while the frequency of two-hit chromatid aberrations is greatly reduced by fractionation of dosage. Evidence from the analysis of types and proportions of chromatid aberrations induced during the prophase cycle shows that a single hit may break one or both of the sister chromatids. Division figures analysed at intervals between 3 and 25 hours after irradiation show only chromatid aberrations. During this period the double deletions, caused by breaks in both chromatids by a single hit, decrease in frequency as the distance between sister chromatids increases. The single deletions and exchanges, which involve breaks in only one of the two sister chromatids, increase in frequency as the sister chromatids separate during prophase development.

The production of breaks in both sister chromatids is supported by the analysis of ultraviolet effects (Swanson, 1940). Irradiation of the generative nuclei in *Tradescantia* pollen tubes with X-rays produces single and double chromatid deletions in

TABLE 8. EFFECT OF FRACTIONAL DOSAGE ON FREQUENCY OF CHROMOSOME ABERRATIONS
5-day

X-ray intensity	No. of exposures	Duration of exposure	Rest periods	Total Chr.	Percent breaks $2(D+R)/n$
40r/m	1	4'	0	4800	4.1
	4	1'	20'	5400	1.6
	4	1'	40'	4800	1.8
	4	1'	60'	4758	2.4
	4	1'	80'	4800	2.2
	4	1'	120'	2754	2.3
	4	1'	240'	1536	2.2
	1	1'	0	7200	0.4
40r/m					B=1.6
	1	8	0	3600	11.4
	4	2	60'	3600	7.4
	4	2	120'	3600	8.4
	4	2	240'	1158	6.6
180r/m(D)	1	2	0	4200	1.7
					B=6.8
	1	2	0	3600	17.7
	4	0.5	30'	3600	9.1
	1	0.5	0	3600	2.1
					B=8.4

about equal proportions, as well as some chromatid exchanges. Irradiation with ultraviolet produces single chromatid deletions almost exclusively. This evidence indicates that the chromosomes were effectively split when irradiated and that the ultraviolet hit rarely breaks more than a single thread while an X-ray hit can break one or both of the sister chromatids. This interpretation is in accord with Stadler's results with *Zea* (Stadler, 1939). The evidence also suggests that breaks induced by ultraviolet are usually permanent and that fusion of broken ends rarely occurs.

The proportions of single and double deletions induced by X-rays during prophase development

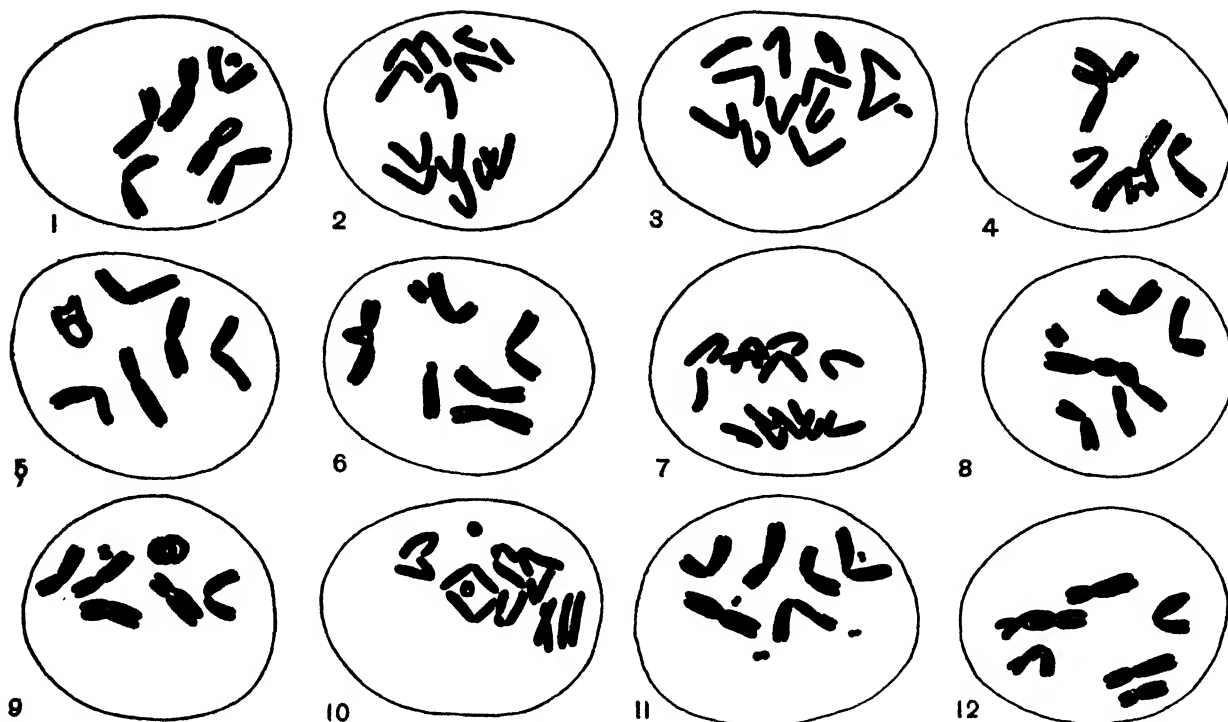


PLATE I—FIGS. 1-12

Camera lucida drawings of the various types of chromatid and chromosome aberrations in *Tradescantia* microspores following X-ray treatment.

FIG. 1. A single and a double chromatid deletion; FIGS. 2 and 3. The same at anaphase; FIG. 4. The two types of chromatid exchange between different chromosomes; FIG. 5. Chromatid ring; FIG. 6. Chromatid buckle and a terminal deletion; FIG. 7. Terminal deletion followed by free separation of centric chromatids and partial fusion of fragments; FIG. 8. Dicentric chromosome and acentric fragments; FIG. 9. Ring chromosome and acentric fragments; FIG. 10. Ring and dicentric at anaphase; FIG. 11. Interstitial deletions; FIG. 12. Unequal translocation.

show that the sphere of influence of a single hit is relatively large, but it is usually insufficient to include both sister chromatids at late prophase. The dosage curves for chromatid exchanges indicate that a single hit rarely, if ever, breaks chromatids of two chromosomes. The great majority of breaks must be so isolated that no illegitimate fusions can occur. The breaks producing aberrations must constitute a very small fraction of the original breaks induced by X-rays.

Some terminal deletions are produced by X-rays and the broken ends of the centric chromosomes appear to behave like normal ends. Such deletions are much less frequent following X-ray treatment than are induced by ultraviolet radiation, indicating that the two types of radiation are quite different in their effects.

The relation between frequency of two-hit aberrations and X-ray dosage is dependent upon radiation intensity. When the dosage is given at high intensity the aberration frequency increases approximately as the square of the dosage, even when the dosage is varied by varying the time of exposure. At lower intensities the aberration frequency follows the square law at low doses because the duration of

exposure is too short to permit the operation of the time-intensity factor, but at higher doses and longer exposure periods the exponent of the dosage curve equation declines until it approaches 1.0 at 2.7 r/m (fig. B). The aberration frequency would be expected to increase as the square of the dosage if each of the aberrations is caused by two independent hits. The nearly linear dosage curve at 2.7 r/m is based on the same types of aberrations which presumably are dependent upon two independent breaks. But due to restitution during irradiation with long exposure times fewer breaks are available for the production of dicentric and ring chromosomes. These aberrations become more dependent on the occurrence of two breaks within the critical time period as exposure time is increased. Although two breaks are necessary to produce the aberration, the occurrence of the two breaks within the critical limits of time and space must be considered from the statistical standpoint as a single event. Such an assumption would account for the slope of the dosage curves at low X-ray intensities, but whether such an explanation will satisfy the biophysicists is a question which might well be discussed at this symposium.

The evidence regarding the duration of open breaks is conflicting. The fractional dosage experiments indicate that practically all of the breaks undergo restitution or illegitimate fusion within an hour at most and probably in a much shorter period. The evidence from the dosage curves indicates about an hour for breaks induced at 2.7 r/m and about 15 minutes for breaks induced at 20 r/m. Exposures given within 3 minutes produce only slightly higher aberration frequencies than exposures covering 6 minutes, indicating that fusion of breaks is not immediate. The duration of the transition from chromatid to chromosome breaks is about 4 hours. If chromosome aberrations,—dicentric and rings—can be derived only from breaks in unsplit chromosomes, the evidence would suggest that chromosome breaks could remain open for several hours, but if both chromatid and chromosome aberrations can be derived from breaks induced just after effective splitting, the evidence from the transition period is of little value. The evidence from fractional dosage seems to be the most critical and indicates that the period between breakage and fusion is considerably less than an hour.

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SUMMARY

Irradiation of *Tradescantia* microspore chromosomes with X-rays produces only chromatid aberrations during the first 25 hours after raying, when the period of nuclear cycle is at a minimum. Between 27 and 31 hours both chromatid and chromosome breaks are found in considerable numbers, and from 34 hours to 5 days after raying only chromosome aberrations appear in the division figures. Single chromatid deletions are most frequent shortly after raying when the sister chromatids are further apart at late prophase, while double deletions are most frequent following irradiation of early prophase when the sister chromatids are closely associated. However, a single hit can break both sister chromatids throughout prophase, even when the chromatids are easily differentiated in microscopic preparations.

The relation between X-ray dosage and aberration frequency depends on the nature of the aberration and the intensity of the radiation. The relation between dosage and the frequency of one-hit aberrations is linear regardless of the time-intensity factor. With short exposure times, the two-hit aberrations increase in frequency as the square of the dosage, but as radiation intensity is decreased the aberration frequency declines and the dosage curve approaches linearity at 2.7 r/m. The effect of the time-intensity factor is due to restitution of broken ends so that fewer adjacent breaks occur within the critical limits of time and space.

The evidence from varying intensity and from fractional dosage indicates that breaks can remain open for only 26-60 minutes. Due to limitations in

time and space only a small proportion of the original breaks must be involved in the production of aberrations. Broken ends usually fuse and if illegitimate fusion does not take place, restitution occurs so that there is no evidence of previous breakage.

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DISCUSSION

MULLER: Are you sure of the accuracy of the conclusions from the ultraviolet results?

SAX: Our conclusions are purely tentative. Swanson and Uber are still working on the experiments. It is very difficult to grow the pollen tubes.

ZAMENHOF: In X-radiation, one hit produces one break; in ultraviolet radiation, we are not yet sure of this. There is evidence in the German literature that two hits are necessary to produce a break in radiation with ultraviolet. Actually every locus receives many thousands of hits with ultraviolet.

SAX: Muller has disposed of the sensitive volume argument primarily because it must be assumed for every determination 1) that every hit is effective, and 2) that you see every effect which is produced.

HOECKER: May it not be that in the case of X-rays, the energy of a quantum is sufficient to break both strands when their plane is parallel to that of the incident radiation, while in ultraviolet it might not be sufficient?

SAX: This may occasionally occur, but it is improbable that the secondary radiation would often hit the second thread.

RS: From X-radiation of coccids, Mrs. Schrader

and I have evidence that four strands are broken by one hit, since it is improbable that single hits would occur four times in one place. In late prophase, the stage irradiated, the chromosome consists of four coiled strands running parallel. Chromosome breaks at metaphase are frequently observed.

DELBRÜCK: In the neutron experiments, was the time or the intensity of the irradiation kept constant?

GILES: In the majority of the neutron experiments with *Tradescantia* the time of irradiation has been kept constant; in a few, higher doses were obtained by increasing the time. The relation between the percentage of breaks and the dosage for chromosome exchanges in all the experiments so far as has been approximately linear.

FANO: The action of X-rays is straggled in a known manner along the path of secondary electrons, so one may check the wave length at which straggling becomes important in relation to the thickness of the chromosome.

SAX: Rick and Fabergé have found no wave length effect. We have run dosage curves at two and a half million volts and get the same results as that given by the usual X-ray machine.

MULLER: (to Delbrück) What is the radius of a cluster of ions?

DELBRÜCK: A few Ångströms.

MULLER: This distance is too small to be of help in explaining a spreading of the effect of one hit. But further evidence concerning such spreading might be obtained through a study of the comparative morphology of the rearrangements produced by irradiations of different intensities, because proximity might be more effective under some circumstances than others.

SAX: Probably not because the proximity may be less than $\frac{1}{2}$ micron.

SCHULTZ: Has the relation of aberrations at early prophase to those at late prophase been examined? The effect of the radiation may become evident when syntheses are taking place in the chromosome. The neutron effect would thus be more important than otherwise.

LURIA: Experiments with soft X-rays (about 15 kilovolt for example) could give very different results from those with X-rays of 100 kilovolt.

SAX: Is the killing relation then linear?

LURIA: The effect would approach that of neutron experiments.

DELBRÜCK: With reference to Sax's point that X-rays are more effective the closer the strands are together, there is no doubt that a break is a single hit event. You imply that this is due to two ionizations along the track of a recoil electron?

MULLER: We must assume one ion, not one recoil electron, to cause two neighboring breaks.

DELBRÜCK: Sax's idea is that with X-rays two sister chromatids can be broken if close together because the recoil electron has then a greater chance of traversing both chromatids.

SAX: It is logical that if the strands are close

together, the recoil electron is more likely to traverse them at close range, but it is more probable that a single ionization is involved.

MULLER: We cannot get them close enough in *Drosophila* to be hit by the same recoil electron as frequently as by different electrons.

DELBRÜCK: Then the whole argument falls down for X-rays.

MULLER: No, the effect of one ion spreads. One ionization, produced by one hit, can break two points as shown by minute rearrangements in *Drosophila*.

DELBRÜCK: We must ask which is more sensitive to radiation, the capacity of a gene to produce a character by enzymatic action or its capacity to reproduce. I now think the latter is more sensitive, because in the case of viruses and phages, the sensitive volume is nearly as big as the true volume of the self-reproducing unit. Therefore these analyses should take account of the autonomous biochemistry of the gene. We observe always at least part of one cell cycle later than we irradiate. Irradiation probably interferes with the autonomous biochemistry. I am still skeptical that one ionization affects two visibly separate chromatids at identical loci. It is understandable that it spreads along one chromatid. But if the threads are already separate, one would think that their biochemical activity was also separate. Ris' case makes me more suspicious still that we are on the wrong track. Who knows how separate the strands are before they are fixed and stained?

FANO: If ionization interferes with the biochemistry of the threads, then many things can happen subsequently as a secondary effect of the biochemical phenomenon. For example, another chromatid might be broken in the neighborhood.

DEMEREK: In case of the translocation N264-86, induced by X-rays in a *Drosophila* male, two strands of the treated X chromosome were recovered. One of them has a deficiency from 3C8 to 3E5, inclusive, and in addition carries changes in *fa* and *rst* loci to the left of the deficiency, while a piece from 3C7 to 3E5, inclusive, has been taken out from the other strand and inserted into the chromocenter of 4. Thus the right breaks occurred in both strands at the same point, while the left breaks occurred at different points but close to each other. It has been interpreted that the left break was brought about through the action of a single excitation shock given by an electron to the surrounding matter and diffused over a relatively large area covering two strands in width and about 150 Å in length.

SAX: (called on D. W. Bishop in connection with the hit and break idea).

BISHOP: In *Chortophaga* spermatocytes, breaks in one thread or two threads of diakinesis chromosomes can be produced within 30 minutes after treatment with 200 r.

DELBRÜCK: Sax's effect in 20 minutes is even more convincing.

STADLER: How positive is the evidence that the

chromosome rayed before two breaks were obtained was already double?

NEBEL: It is positive but optical; we are sure of the stage rayed, and the optical duplicity exists earlier in the cell cycle.

STADLER: In corn pollen treated with ultraviolet and X-rays, the predominant effect of ultraviolet seems to be on the half chromosome, that of X-ray on the whole chromosome. Yet the treatment is applied at precisely the same stage. We might assume either 1) that the chromosome is predivided and that X-rays usually affect both strands together, or 2) that the chromosome is not divided, and the radiation effect on a single unit may result in a change which will affect both daughter units in some cases and only one of the daughter units in other cases. With X-rays the change affects both daughter units much oftener than with ultraviolet. In corn,

there is no cytological evidence which requires us to adopt the first assumption. In view of the physical considerations, the second assumption seems the more plausible.

NEBEL: I think we must consider locus-specific connections between chromatids during interphase, even if the chromatids are microscopically separate.

RIS: That the coccid chromonema is separated at least into two threads in the treated stage can be seen in the living cell and is not an artifact.

KAUFMANN: The same argument applies to Bishop's case, since the structure of the chromosome in diakinesis has been checked by ultraviolet photography and by Belar's observations on the living cell.

MULLER: Breakage at diakinesis might occur by a mechanism similar to that of crossing over; if so, the occurrence of a second break would be correlated with that of a first break.

EFFECTS OF X-RADIATION ON GRASSHOPPER CHROMOSOMES

J. GORDON CARLSON

Few animal cells are better suited to certain kinds of cytological investigations than the spermatogonia, spermatocytes, and neuroblasts of the grasshopper. Of particular advantage are the large sizes of both cells and chromosomes, their typical mitotic and meiotic behavior, the ease of preparing them for study, and the ready availability of the material.

The germ cells are arranged within the follicles of the testis in the order of their formation and development. At the distal or free end are primary

The neuroblasts of the embryo constitute the most favorable somatic cells for cytological study. These are groups of large cells situated on the ventral surface of the embryo on either side of the mid-ventral line of the thorax and abdomen and on parts of the ventral and anterior regions of the head. Compared with the spermatogonium, the neuroblast and its nucleus are considerably larger and the chromosomes are more widely spaced and therefore more easily studied. The neuroblast divides very unequally to give rise to a daughter neuroblast and daughter ganglion cell. Once the neuroblasts are all laid down, therefore, their number remains relatively constant. This is an advantage in statistical studies not possessed by cells that double in number with each generation. Neuroblasts are easily prepared for chromosome studies by fixing and staining the whole embryo in aceto-carmine one to two hours, placing it on a glass slip in a mixture of two parts of aceto-carmine and one part of karo syrup, and separating the cells by tapping the cover glass sharply. In such a smear the neuroblasts can be readily distinguished from the other cells by their large size and characteristic shape.

Recently a method of observing neuroblast mitoses directly in the living state has been worked out. Grasshopper embryos of a suitable age are dissected out of the egg membranes and oriented in a hanging drop of artificial culture medium with the ventral side of the body upward against the cover glass. This brings living neuroblasts within range of the highest powers of the microscope. By this method it is possible to distinguish thirteen separate phases of the mitotic cycle, which are shown in Figure 1. The approximate relative durations of the recognizable phases for the artificial culture medium at 26° C. are indicated, the whole cycle averaging about eight hours. The stages designated may be characterized briefly as follows. *Prophase*: very early—nucleus containing barely visible chromatin threads in addition to the highly refractile granules characteristic of the preceding interphase; early—initiated by the disappearance of the nuclear granules, the chromatin threads becoming more distinct as the cell progresses; middle—the chromatin threads thick enough to be traceable from one part of the nucleus to another by careful focussing; late—initiated as the chromatin threads become sufficiently short and well spaced that about seven can be counted near the nuclear membrane in 1/4 of its circumference in mid-optical section (includes the critical period); very late—initiated by breakdown of the nuclear membrane. *Metaphase*: the chromosomes located throughout their lengths

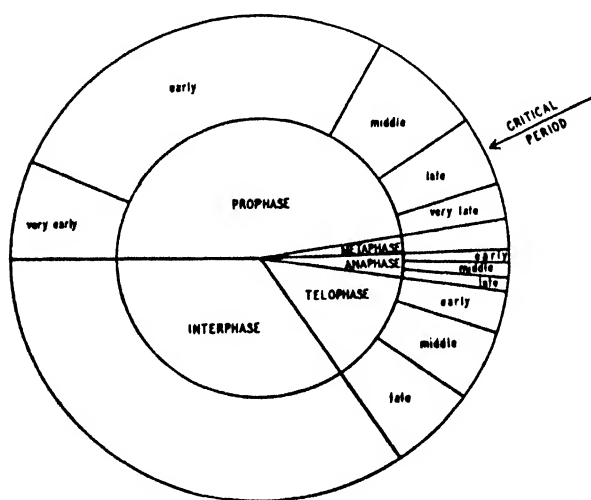


FIG. 1. A diagram indicating the relative durations of the different stages in the mitotic cycle of neuroblasts of *Chortophaga* in artificial culture medium at 26° C.

spermatogonia, then, in order, secondary spermatogonia, first spermatocytes, second spermatocytes, spermatids, and finally spermatozoa, which occupy the proximal or attached end. Beginning with the secondary spermatogonia the cells are grouped in cysts. All the cells of a cyst have been derived by successive divisions from a single primary spermatogonial cell. At the end of the secondary spermatogonial divisions—six or seven in most of the commonly studied species—each cyst contains 64 or 128 cells, respectively. This is quadrupled by the two meiotic divisions. All the cells of a cyst pass through the division stages synchronously, a characteristic that makes it possible to confirm particular chromosomal configurations by observing them in several cells. Testis cells may be prepared for study by either section or smear technics, but the latter sacrifice the advantage of the orderly arrangement of the cells and cysts.

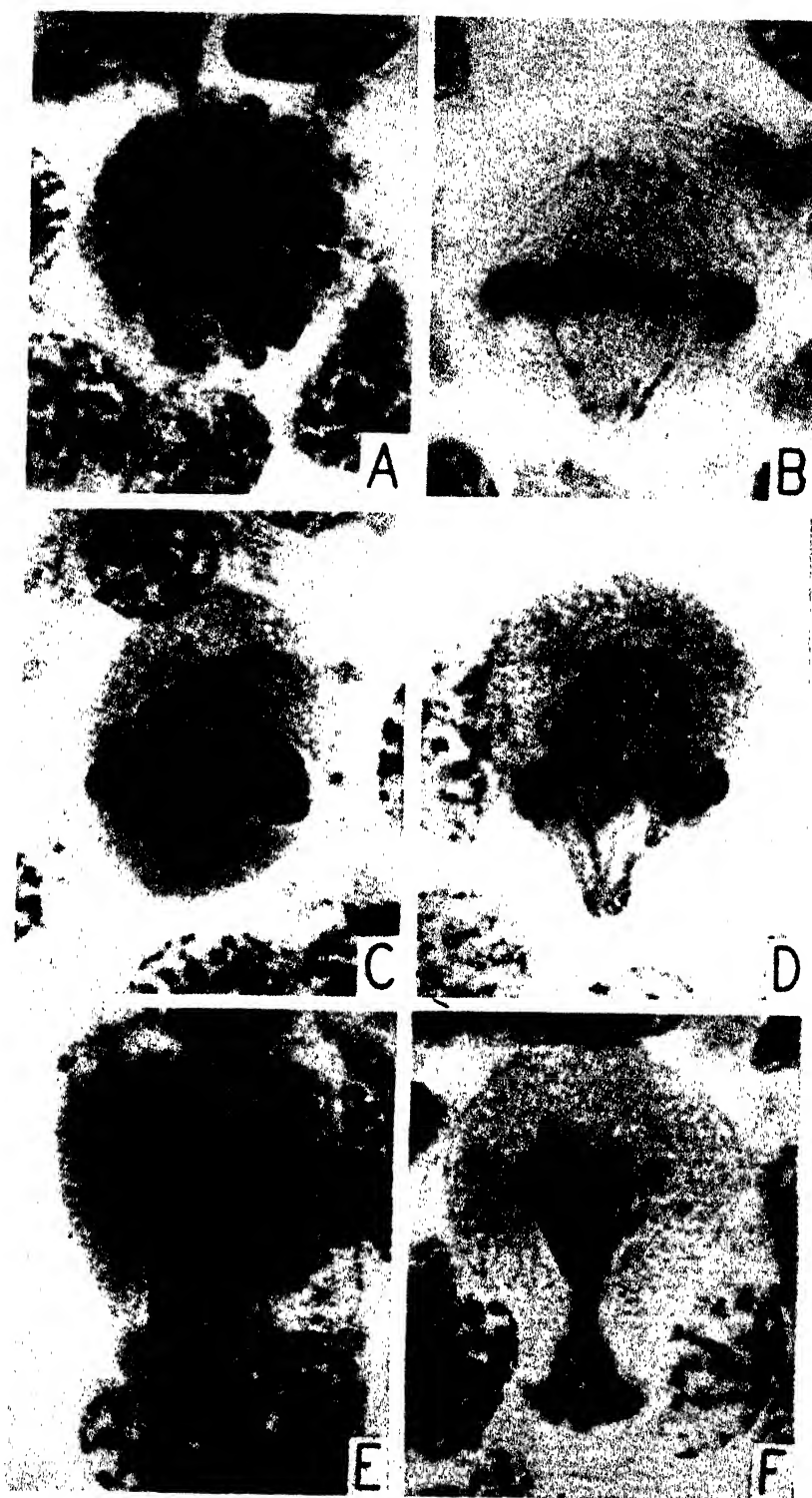


FIG. 2 (see facing text page for legends)

in the equatorial plane of the cell. *Anaphase*: early—separation of sister chromatids beginning at their extreme proximal ends; middle—initiated as the distal ends of the longest daughter chromosomes begin to leave the equatorial plane; late—begins as the cleavage furrow reaches the spindle. *Telophase*: early—introduced by the completion of cytokinesis and the loss of the sharp outlines of the chromosomes; middle—the nucleoli become visible, increase in size, and the nuclear membrane forms; late—initiated as the nucleoli lose their regular spherical shape. *Interphase*: the highly refractile granules representing the remnants of the chromosomes are scattered throughout the apparently homogeneous karyolymph with no observable linear arrangement. All these stages are identifiable in living cells at a magnification of 670.

PRIMARY EFFECTS

If the results of analyses of X-ray induced chromosomal aberrations are to be interpreted correctly in a tissue in which all of the cells do not go through mitosis synchronously, or in which the mitotic stage at the time of treatment is not determinable, it is necessary to know the effects of the X-rays on the different stages of mitosis. An approach to this problem has been made through statistical studies involving a comparison of the relative numbers of certain different stages of neuroblast mitosis in untreated and in X-rayed material at 15 minute intervals following treatment (Carlson, 1940 and unpublished). Additional data on this effect has been secured by observing selected neuroblasts in hanging-drop preparations before and after X-raying. Since the times required for untreated cells to pass through the different mitotic phases is remarkably constant for similar culture conditions at 26° C., the effects of X-rays on each stage can be easily determined.

The exact mitotic stages of certain selected neuroblasts in hanging-drop preparations are recorded, the preparation is X-rayed, and then the effects on each of these cells is determined by direct, continual observation. Such studies have shown that the late prophase is the most sensitive part of the mitotic cycle, at least, as regards the X-ray effect on mitosis. Within this stage is a particular brief period previous to which X-radiation will cause a cell to stop or even to revert mitotically, and following which a cell will proceed through the succeeding metaphase and anaphase with some or no delay, depending on the dosage and the exact stage in which it is x-rayed.

For convenience this will be referred to as the "critical period." It is the stage passed by a normal, untreated neuroblast about 10-15 minutes (at 26° C) before the breakdown of the nuclear membrane, when the chromosomes are large and almost completely formed.

Cells that have passed this critical period but still retain the nuclear membrane at the time of treatment may show a delay up to 20 minutes in the breakdown of the nuclear membrane after 250 r. The amount of delay is directly related to the nearness of the cell to the critical period; the more recently the cell has passed through the critical period at the time of treatment the greater the delay. Accompanying this delay is a chromosome effect evidenced by the failure of the chromosome halves to separate normally at the succeeding anaphase. The non-proximal portions of the daughter chromosomes tend to cling together so tenaciously that the longer elements may not separate completely up to the time of cleavage and the reconstitution of the daughter nuclei. The severity of this phenomenon is directly related to the previous delay in the breakdown of the nuclear membrane and therefore to the stage of the cell at the time of treatment. In extreme cases, where there is a delay of about 18-20 minutes over the normal in a cell treated immediately after it has passed the critical stage, the metaphase chromosomes have a tendency to stick together distally in groups, and at late anaphase the whole mass of closely grouped chromosomes, which cling together distally, has the form of an hour-glass. This is eventually severed at its narrowest place by the cleavage furrow, and a daughter nucleus is reconstituted about each of the chromatin masses, which gradually undergo diffusion beginning at the poles and extending toward the plane of cleavage. The degree of this effect is also related to dosage. After the relatively high dose of 8000 r, for example, the chromosomes become clumped together at metaphase in the form of a solid ring-shaped mass, the daughter halves of which move toward opposite poles at anaphase far behind the kinetochores with which they maintain a connection through long, attenuate chromatin threads (fig. 2). Cells treated in late prophase after the nuclear membrane has broken down show none of these effects. White (1937) has described what is probably the same phenomenon in the first meiotic division of *Mecostethus* and *Stauroderus*. The chromosomes of first spermatocyte metaphases $7\frac{1}{4}$ hours after X-raying appear normal, but the daughter chromosomes at anaphase cling together distally. If the effect is not

FIG. 2. Photographs of Chortopaga neuroblasts fixed and stained in acetocarmine 15 minutes after the end of treatment with 8000 r at a rate of 200 r per minute, showing the primary effect of X-radiation. A—very late prophase, the chromosomes irregular in outline and fused with one another in certain regions; B—early anaphase, the kinetochores near the poles but connected with the main equatorial chromosomal mass by long, attenuate chromatin threads; C, D, E—successively later anaphases, the equatorial chromosomal mass gradually following the kinetochores in the movement toward the poles; F—early telophase, cytokinesis complete and the chromosomal masses concentrated toward the poles. $\times 1000$.

extreme, separate interkinetic nuclei result, but if separation is incomplete, a single restitution nucleus containing both groups of daughter chromosomes is formed. This effect appears several hours after irradiation in these cells as contrasted with about one hour in neuroblasts. This apparent discrepancy is probably not significant, however, for the meiotic prophase is much longer and more complex than the neuroblast prophase. There is nothing in my material comparable to the chromosome disintegration found by White in these same cells 25 hours after X-raying, which may be a special phenomenon produced by treatment of spermatocytes at a special prophase stage that is lacking in the neuroblasts.

The presence of cells with clumped chromosomes shortly after irradiation has been noted by many investigators, who have usually referred to it as the "primary effect" of X-rays (Alberti and Pollitzer, 1923) and ascribed it to induced physiological changes, in contradistinction to "secondary effects," which supposedly result from direct "hits" on the chromosome to give chromosomal aberrations of genetic importance. It is generally assumed that the pycnotic cells appearing in tissues many hours after X-raying are the fatally injured cells in which this clumping has occurred.

When neuroblasts in the phase just preceding the critical period are treated with very low X-ray doses, such as 8-16 r, progressive changes in the chromosomes gradually slow up and then cease for several hours. Cells in earlier prophase continue to advance, though more slowly than normal. As a result cells tend to accumulate in middle and late prophase stages. As recovery takes place, the original mitotic order of the cells is altered. Cells that were treated in middle prophase reach metaphase first, then those that were just previous to the critical period, and finally, in irregular order, those that were in interphase and early prophase. This shuffling of the original order may account in part for the broad overlapping in chromatid and chromosome effects found by numerous investigators in cells that have reached metaphase or anaphase simultaneously.

After somewhat higher doses, for example, 250 r, the chromosomes of cells approaching the critical period continue the progressive development typical of late prophase for a few minutes, then slow up, stop, and gradually begin to revert to a condition resembling the early prophase stage. This occupies about an hour. Such cells, together with those in early prophase at the time of treatment and those which advance from interphase after treatment, remain at this stage for many hours. With recovery, all these cells slowly progress into metaphase and anaphase stages. To what extent there is an altering of the original mitotic order as at the lower doses has not yet been determined.

SECONDARY EFFECTS

These cells, which have been delayed in prophase for a period of time varying with the dosage, exhibit

at metaphase and anaphase the structural chromosome changes sometimes known as secondary effects (fig. 3). The classification below includes the main kinds of fundamental changes, but at higher doses two or more of these may involve the same chromosomal elements.

Fragments. The simple break of a chromosome results in two fragments, the one possessing and the other lacking a kinetochore. The sister chromatids of these elements fuse at their broken ends to give dikinetid and akinetic chromatids, respectively. The mitotic behavior of these fragments in neuroblasts of *Chortaphaga* has been described (Carlson, 1938).

At metaphase the akinetic fragments are distributed irregularly in the equatorial plane of the cell near its periphery. Their chromatids undergo anaphase separation simultaneously with the non-proximal portions of the unaltered chromosomes. If such a fragment is terminal, it opens out first as a V, the arms of which finally break at the place of fusion to give two rods of equal size. The intercalary fragment, owing to fusion of sister chromatids at both ends, opens out first as a ring, then with breakage at one point of fusion it assumes the form of a V or J. It is not known to what extent breakage at both points of previous fusion may occur to give two rods. Akinetic fragments with chromatids that open from the first as completely separate rods may have arisen from the fusion of the broken ends of two such fragments to give a single element with two true ends, in which case they would, in a sense, be chromosome translocations. The sister chromatids of akinetic fragments move toward opposite poles at late anaphase and frequently come to lie in different daughter cells by telophase. In one embryo daughter halves of at least 63 percent of the akinetic fragments were situated in different daughter cells. If these fragments lie close to the telophase group of chromosomes at the time of nuclear membrane formation, they may be included within the main nucleus; if not, they occupy smaller, accessory nuclei in the cytosome.

Kinetic fragments are indistinguishable at metaphase from normal chromosomes. At anaphase they form chromatin bridges. That this is not a strong and permanent union, however, is evidenced by the fact that in almost all of these breakage of the bridge appears to occur exactly in the middle of the element and therefore at the point of former fusion. Occasional failure to separate at the point of fusion may be due in some cases, at least, to breakage at a weaker point, and this leads, of course, to a deficiency in one daughter cell and a duplication in the other.

The apparent absence of chromatid fragments in metaphases following irradiation and their infrequency even in anaphases of neuroblasts may be due to the close contact between sister chromatids up till late metaphase, a state that is conducive to partial or complete rehealing of a broken chromatid

before the akinetic fragment becomes free at anaphase. Possibly the X-ray induced constrictions present at metaphase and anaphase mark the sites of incomplete or partially refused chromatid breaks.

Chromatid Translocations. Two main kinds of chromatid translocations are present in neuroblasts of the grasshopper (Carlson, 1938a). In the one type the proximal portion of the affected chromatid of each chromosome becomes joined to the distal portion of the affected chromatid of the other chromosome to give a figure resembling a cross-shaped tetrad with a chiasma. Each of the four chromatids has a single kinetochore; and the orien-

not feasible in *Chortophaga* to attempt to identify in the unsynapsed condition chromosome translocations of the type in which mutual exchanges of distal or proximal ends have occurred. That they do occur is indicated by the occasional presence after treatment of abnormally long chromosomes in neuroblasts of *Chortophaga* (Carlson, 1938a) and by the synaptic configurations analyzed by Helwig (1933, 1938) in the first meiotic metaphase of *Circotettix*. Translocations in which distal and proximal ends become joined *inter se* are easily identified in neuroblasts. The dikinetid element has both ends on the spindle, while the akinetic part

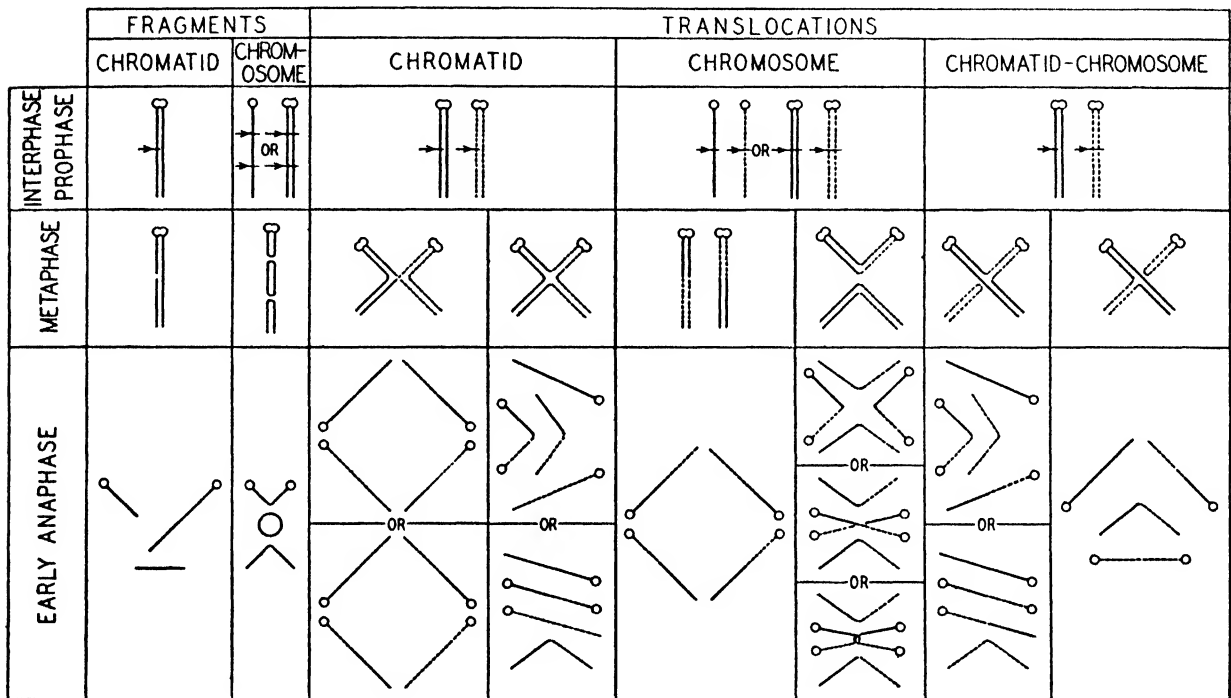


FIG. 3. Diagram of the main types of X-ray induced chromosomal changes at metaphase and early anaphase in neuroblasts of *Chortophaga*. The horizontal arrows in the interphase-prophase stages indicate effective X-ray "hits."

tation on the metaphase spindle will determine whether each or neither of the daughter cells will have a duplication and deficiency. In the other type the two proximal and the two distal portions of the affected chromatids of the two chromosomes are joined *inter se*; as a result one of the reconstructed chromatids is dikinetid and the other akinetic. Depending on its orientation on the spindle, the dikinetid chromatid may either pass completely to one pole or form a chromatin bridge at anaphase. Out of 86 chromatid translocations produced in neuroblasts by doses of 125, 250, and 375 r it was possible to determine accurately the type of only 14; 5 of these were the chiasma-like and 9 the dikinetid type.

Chromosome Translocations. Because of the relatively large number of chromosomes per cell it is

lies near the periphery of the cell adjacent to the dikinetid element. The presence, not infrequently, of two instead of one akinetic element in this region suggests that the distal parts are not invariably fused. Depending on the orientation of the dikinetid element on the spindle at metaphase, separation of the chromatids at anaphase may occur in such a way that 1) a U-shaped dikinetid daughter chromosome passes to each pole, 2) two crossed chromatin bridges are formed as a result of a half turn of the chromatids about one another, or 3) the U-shaped daughter chromosomes are interlocked owing to a full turn of the chromatids about one another. Of 13 neuroblasts in which it was possible to analyze accurately the type of separation, nine were free U-shaped, five were crossed chromatin bridges, and one was interlocked U-shaped. This

would be expected on a purely mechanical basis.

Combination Chromatid-Chromosome Translocations. Besides exchanges that involve chromatids or chromosomes exclusively there is a class in which the ends of two broken chromatids representing either the distal or proximal portion of one chromosome are fused with the broken ends of the distal and proximal portions of one chromatid of another chromosome to give a branched element at metaphase (White, 1935; Carlson, 1938a). If the chromosome contribution is a proximal end, the newly constituted chromosomal element will consist of one dikinetid and two monokinetid chromatids. At anaphase this dikinetid element will either go to one pole as a U-shaped daughter chromosome or form a chromatin bridge. If the chromosome contribution is a distal end, the new chromosome will consist of three chromatids, two with a single kinetochore each and one that is akinetic.

Chromosome Constrictions. The constrictions that result from X-radiation may represent partial breaks or incomplete fusions. They range from very slight indentations to constrictions so deep that the parts on either side are joined only by slender threads. Because of the presence of normal secondary constrictions on certain chromosomes with which these induced ones can be confused and because of the great range in the depth of the constriction, they are very difficult to count and classify for statistical analysis.

Extreme Changes After High Doses. As the X-ray dose is increased above 250 r, the number of fragments and translocations per neuroblast also increases. At 500 r the changes are so numerous and complex as to be analyzable only with great difficulty. From 1000 r up to 6000 r the effects on the chromosomes are so drastic that, instead of individual chromosomes at metaphase, there is a ring-shaped mass of chromatin that is transformed at anaphase into a spindle-shaped mass. At telophase this assumes the shape of an hour-glass, which is finally severed at its middle by the cleavage furrow. This resembles the primary effect after high dosage as illustrated in Figure 2.

TRANSMISSIBILITY AND PERSISTENCE OF SECONDARY EFFECTS

The structural changes so far considered are those observed in later stages of the same mitotic cycle in which they were produced. Of importance from the cytogenetical point of view is the question of the fate of these changes in subsequent cell generations. To what extent do these change from cell generation to cell generation and what is the mechanism of this change?

On the basis of their mitotic transmissibility the previously-described aberrations fall into three classes:

1) **Non-transmissible Aberrations.** These by their very nature have a different expression in the second

cell generation. Chromatid effects are in this category, since the chromatid fragments and translocations of the first cell generation become the chromosome fragments and translocations of the next generation. These will then fall into one or the other of the next two groups.

2) **Unstable Aberrations.** The expression of the instability of chromosome aberrations depends on the orientation of the chromosomes on the spindle and the capacity of broken ends of chromosomes to heal or fuse with other broken ends for more than a cell generation after their production. This class includes chromosome fragments and those translocations that result in one or more dikinetid chromatids, if these are oriented on the spindle in such a way as to form chromatin bridges at anaphase.

3) **Stable Aberrations.** Chromosome translocations in which none of the chromatids is dikinetid are permanently stable from the first, and so will not become altered through the normal operation of the mitotic process.

Altered chromosomal elements that are mitotically transmissible should persist indefinitely provided they are not cell lethal to any particular generation of cells.

A comparison of chromosome aberrations in spermatogonia and neuroblasts in the first and subsequent cell generations following their production would be expected to provide evidence on the ultimate fate of different aberrations. Two serious, potential sources of error, however, are involved in such studies: the difficulty of determining accurately 1) the number of mitoses intervening between treatment and study of a given cell and 2) the extent to which elimination of cells and the aberrations they contain has occurred as a result of lethal effects. The use of neuroblasts in culture for direct observations of aberrations in living cells may overcome these difficulties. The present, scanty evidence is considered as it applies to the unstable aberrations.

Akinetic Fragments. White (1935) found akinetic fragments in spermatogonia of *Locusta* as late as 14 days after irradiation. He believed that such cells were in the fourth division following treatment. Carothers (1940) has reported the presence of such fragments in somatic cells of the embryo of *Melanoplus* seven days after X-raying, and I have found them in *Chortophaga* neuroblasts as many as 11 days after treatment. Despite the mitotic delay occasioned by X-raying, these would seem to be several generations removed from treatment, since a single mitotic cycle normally requires in cultured neuroblasts but eight to ten hours. Have these fragments persisted as such through several mitoses, are they the consequence of delayed breakage resulting from previous treatment, or are they merely later manifestations of half, quarter, eighth, etc., chromatid effects induced at the time of irradiation? The evidence is much too meager to provide an

answer to this question at the present time, but none of these appears to be eliminated as a possibility.

Both White (1935) and Carlson (unpublished) have found a gradual decrease in the mean number of akinetic fragments per cell in spermatogonia and neuroblasts, respectively, following the mitotic cycle in which they are produced. In the spermatogonia this might well be due to the elimination of the cells with the larger numbers, since White states that 24 hours after treatment large numbers of pycnotic nuclei were present. In the neuroblasts, on the other hand, there seems to be no appreciable elimination of cells through pycnosis, at least after a dose of 250 r, which produces a comparable average number of such fragments per cell in the first division after treatment. Disintegration of those fragments not included in the main nucleus at telophase or delayed reattachments of the broken ends of akinetic and kinetic fragments would account for this decrease, but the actual occurrence of neither of these has been proved.

Kinetic Fragments. Chromatin bridges resulting from fusion of sister chromatids have not been found in anaphases of neuroblasts a sufficient length of time after treatment to provide any assurance that the cell was in its second or a later division. Their absence might be due to 1) healing of the broken ends, 2) delayed attachments with akinetic fragments, or 3) elimination of the cells containing them. As yet there is too little evidence from studies of the grasshopper bearing on this point to justify any conclusions. Evidence from other organisms indicates that healing of broken ends may occur in some tissues and not in others, e.g., maize (McClintock, 1941), that healing may occur rarely, e.g., *Drosophila* (Sutton, 1940), or that the mitotic stage of the cell at the time of treatment may determine whether healing or fusion of broken ends occurs, e.g., *Tradescantia* (Sax, 1940).

NUMERICAL CHROMOSOME CHANGES

Diplochromosomes. The term "diplochromosomes" was first used by White (1935) to designate X-ray induced V-shaped chromosomes possessing twice the normal number of chromatids at metaphase. They were found in one to ten percent of the spermatogonial divisions of five individuals of *Locusta* fixed 3 to 11 days after X-raying. He believed these were due either to an extra division of the chromatids between metaphases or to a failure of the spindle attachment to divide at prometaphase with the resultant absence of anaphase separation and the inclusion of all elements still attached at their kinetochores in a single nucleus. He suggested that these either may be self-prepetuating or they may eventually break up into normal components to give cells with tetraploid nuclei. Carothers (1940) described similar chromosomes in somatic cells of *Melanoplus* embryos five days after treatment and I have occasionally noted them in neuroblasts of

Chortophaga. It seems not unreasonable to suppose that these diplochromosomes may have originated in cells which were approaching the previously-described critical period of late prophase at the time of treatment, were caused to revert, and then underwent a second doubling process as they progressed toward metaphase the second time.

Heteroploidy. Giant cells containing hundreds of chromosomes and tetraploid spermatogonia and spermatocytes capable of giving rise to giant spermatozoa were reported by Helwig (1933) in the X-rayed *Circotettix* testis. Embryonic cells containing 48 and occasionally 96 chromosomes were found by Carothers (1940), who suggested that they had arisen through complete separation of chromatids at prophase with suppression of the metaphase. She also reported cells with aneuploid complexes of 25, 26, 27, 33, etc., chromosomes instead of the normal 23 and 24 in the male and female, respectively, and suggested that the extra elements in these may originate from diplochromosomes.

LOCUS OF CHROMOSOME BREAKAGE

The only evidence from studies of grasshopper chromosomes bearing on the place of breakage is the study of Helwig (1938), who analyzed induced reciprocal translocations in the chromosomes of *Circotettix* from the synaptic configurations of the first meiotic metaphase. At the time of X-raying the definitive gonad had not been formed and secondary spermatogonia were not present; hence the same chromosome aberrations would be expected to appear in all the cells of a cyst.

He found that the four chromosomes bearing homologous parts as a result of a translocation usually formed a closed-circle, but that in most cysts there were a few spermatocytes with chains instead. Classified on the basis of position of spindle fiber attachment, rings were present in all of 37 cysts in which only atelomitic chromosomes were involved, in 33 of 39 in which two atelomitic and two telomitic chromosomes were involved, and in only three of nine cysts in which only telomitic chromosomes were involved. On the postulate that the forces holding the chromosomes in a circle at metaphase are directly related to the length of the translocated region—as evidenced in the frequent failure of synapsis of translocated pieces involving the smallest chromosomes—Helwig has interpreted these results to mean that breakage occurs more frequently near the spindle attachment than elsewhere; for in an atelomitic chromosome the point of breakage would thus be nearer the middle of the chromosome than in a telomitic one and with the relatively longer regions on either side of the point of breakage the synapsed elements would be less likely to fall apart to give a chain than in the telomitic ones, where one of the elements would be very small.

With regard to the frequency with which chromo-

somes of different sizes were involved in translocations, he found that the largest 12 were involved in 89 percent of the translocations, while the smallest four were involved in only 3.5 percent. He interpreted this to mean that the incidence of breakage is directly proportional to the size of the chromosome.

RELATION BETWEEN DOSAGE AND SIMPLE BREAKS

In *Chortophaga* neuroblasts the percentage of breaks, as measured by the number of fragments produced, is directly proportional to X-ray doses of 125 r and less (Carlson, 1941). This linear proportionality existing between the dosage measured in r units and the number of fragments, the ap-

TABLE 1. PERCENTAGES OF CHORTOPHAGA NEUROBLASTS CONTAINING DIFFERENT KINDS AND COMBINATIONS OF TRANSLOCATIONS AND CHROMOSOME FRAGMENTS AT SEVERAL TIME INTERVALS AT 26° C. AFTER TREATMENT WITH 125 r OF X-RAYS

Hours after X-radiation	12	24	36	48	72	96
Chromatid translocations	12	12	3	1	0	0
Chromatid+chromosome translocations	0	4	0	0	0	0
Chromosome translocations	0	8	15	26	25	13
Chromosome fragments	38	57	43	41	34	17

parent absence of any threshold below which breaks do not occur, and the very close correspondence between the observed and expected distributions of the breaks among the different cells after each of the doses support the hypothesis that the single chromosomal break is the result of a single event—ionization or excitation.

If one assumes that ionizations are responsible for chromosome breakage and disregards the rehealing that probably occurs between breakage and analysis, the number of ionizations per break per metaphase chromosome can be calculated. Though the volume of the metaphase chromosome is probably not the volume of the interphase or prophase chromosome at the time of breakage, the two are doubtless of the same order of magnitude. According to the dosage-breakage relationship found a dose of 2408 r would be required to produce 100 percent breakage or an average of one break per chromosome. In terms of ion pairs this means that in a medium-sized metaphase chromosome with a volume of approximately $10.6 \mu^3$, a mean dose of 51,050 ion pairs would be required on the average to produce one surviving break. This suggests that, unless there is an enormous amount of rehealing, the number of ionizations produced within the chromosome is greatly in excess of those effective in producing breaks. This excess would be greatly reduced if it could be shown that, instead of a single ionization, only the very large ion cluster resulting from a primary electron is effective in inducing breakage; for the percentage

of the larger ion clusters decreases sharply as the number of ions per cluster increases.

CHROMATID VERSUS CHROMOSOME EFFECTS

About 12 hours after treatment of neuroblasts with 125 r of X-rays mitosis returns in sufficient numbers for analysis of chromosomal aberrations. In Table 1 is shown the proportion of cells containing chromatid translocations, chromatid+chromosome translocations, chromosome translocations, and fragments with equal chromatids at 12, 24, 36, 48, and 72 hours. Of the translocation-containing cells the proportion exhibiting chromatid translocations falls off gradually from 100 percent at 12 hours to 0 at 72 hours while that with chromosome translocations simultaneously increases from 0 to 100 percent. The 24-48 hour period when both are present might be due to 1) a shuffling of the mitotic order of the interphase and prophase cells by treatment, 2) the capacity of an X-ray "hit" to translocate either one or two strands of a double chromosome during a large part of the mitotic cycle, or 3) the failure of chromosome doubling to occur in all chromosomes of a cell or all parts of one chromosome simultaneously. That the first possibility does occur has been pointed out previously, but whether it can be held responsible for all of the overlapping of these effects is not known. Support for the second possibility is offered by cells and chromosomes that exhibit both chromatid and chromosome translocations. Also the high percentage of cells with chromosome fragments at 24 hours indicates that a single "hit" may break both strands of chromosomes that behave as double structures in their translocation reactions. The third alternative is possible but seems unlikely in view of the strikingly synchronous behavior of the chromosomes in all other respects during mitosis.

Despite a certain amount of shuffling of the mitotic order of the cells between treatment and their reappearance at metaphase, the data provides a certain general picture. A period exists in the mitotic cycle—possibly in early prophase—when the chromatids of each chromosome are in process of becoming sufficiently distinct from one another to react to X-rays as separate individuals and when the chromosomes are particularly sensitive to the X-ray effects that produce both translocations and breaks. As a result the relative numbers of cells containing chromatid translocations, chromosome translocations, and simple breaks or combinations of these is at a maximum at this time. In cells more advanced at the time of treatment effects on single chromatids may persist as chromatid translocations, while effects on both strands have the form of chromosome fragments. In cells less advanced at the time of treatment only chromosome fragments and chromosome translocations are produced, hence it must be assumed either that the strands are single or so closely joined as to behave as a single unit.

In view of the possibility that half, quarter, eighth, etc., chromatid breaks may be the forerunners of fragments present in the second and later generations after treatment, it seems justifiable at present to look on the interphase chromosome as effectively rather than actually single as far as X-rays are concerned.

In contrast to my results, White (1935) found only chromosome effects in spermatogonia fixed 12-25 hours after irradiation, chromosome and chromatid effects at 32 hours, and only chromosome effects at 51 hours and later. A very drastic and orderly effect of X-rays on the mitotic order of these spermatogonial cells, such that the cells more advanced at treatment were retarded until a large number of cells in earlier stages at treatment had passed through metaphase and anaphase, would seem to be the only means of reconciling these results with mine.

CONCLUSIONS

The greatest promise of the future usefulness of grasshopper chromosomes in X-ray studies appears at present to lie in the combined use of artificial culture and fixation-staining methods in acquiring more exact information on certain fundamental problems of chromosome structure and organization. A given cell treated at a known mitotic stage can be observed and followed in the living state and subsequently fixed for detailed chromosome study at any desired stage of mitosis. Four main problems should be amenable to solution by this means: the determination of

- 1) the exact effects of X-rays on the chromosomes of each of the several stages of mitosis;
- 2) the earliest exact stage at which the chromosomes become effectively double in their reactions to X-rays;
- 3) the source of the akinetic fragments present in the second and later divisions following irradiation, whether through passive transmission from cell to cell, through delayed breakage, or through induced half, quarter, eighth, etc., chromatid effects;
- 4) the ultimate fate of fragments, especially those with broken ends, whether their broken ends heal or undergo delayed attachments, or whether the fragments are gradually eliminated either through disintegration as a result of failure to be included in the main nucleus at telophase or through their cell lethal effects.

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DISCUSSION

HUSKINS: Why are there no chromosomal translocations at 12 hours although there are numerous fragments?

CARLSON: The sister chromatids of the chromosome broken in the 2-strand stage may always fuse with each other at the broken ends rather than with the broken ends of other chromosomes. It is also possible that chromosomes which in their translocation reactions behave as double structures may behave as single structures in their breakage reactions.

FANO: How is the observation of translocations as late as 96 hours after treatment related to the usual eight-hour period of mitosis?

CARLSON: Since the delay in mitosis between treatment and the appearance of the aberrations increases with the dosage, all of the cells are in the first division 24 hours and perhaps even 36 hours after treatment with 125 r. From 48 hours on some of these cells are probably in the second division.

SCHULTZ: Have you followed the behavior of the centrosomes in the critical prophase period? Your results seem related to the breakdown of the nuclear membrane.

CARLSON: I have not yet been able to identify and study the behavior of the centrosome in the living cell, though this could be done in fixed and stained material. The critical period precedes by 10-15 minutes the disappearance of the nuclear membrane, but it seems quite possible that this period might coincide with the initiation of the processes involved in its breakdown.

CHILD: Are there differences in the size of the fragments?

CARLSON: Very great differences. They range from tiny spheres considerably smaller in diameter than the chromosome to large elements almost as long as the longest chromosomes.

RIS: In irradiated coccid embryos, fragments are found after five days in the same number as they are found earlier, and they are still present at 44 days. They are found throughout all embryonic development, since they can go normally through the mitotic cycle. The fragments have a spindle attachment. In spermatogenesis translocations are found but they are never found in the somatic cells.

METZ: Can multiple kinetochores be present?

RIS: The evidence rather points towards a diffuse nature of the spindle attachment, as Schrader suggested in 1935.

LURIA: How definite is the evidence that chromosomes are differently sensitive in different stages?

CARLSON: Treatment at very late prophase, metaphase and anaphase gives no immediate visible effect. The situation at the next division is not known.

SONNENBLICK: Were untreated neuroblasts studied? Were there aberrations in the controls?

CARLSON: There were no fragments or translocations in the untreated controls.

GILES: Are fragments identifiable in either metaphase or anaphase?

CARLSON: Yes.

GILES: Can you tell if fragments result from terminal deletion or translocation?

CARLSON: Up to 125 r, translocations are very infrequent.

GILES: Is it possible that any of these are interstitial and not terminal deletions?

CARLSON: If interstitial, they would appear as rings at early anaphase. I have seen no rings at 125 r and lower. At this dose the average number of fragments per cell is between one and two; the occurrence of two independent breaks in one chromosome would therefore be very rare.

MULLER: Do you think that centric parts of terminally deficient chromosomes form bridges in the next division?

CARLSON: There is no evidence as yet. Although bridges were found three days after treatment with 250 r, there is no certain proof that this was not the first division after treatment.

ON THE ANALYSIS AND INTERPRETATION OF CHROMOSOMAL CHANGES IN DROSOPHILA

U. FANO

Some progress towards a comprehensive understanding of mutation phenomena in *Drosophila melanogaster* has been made by the Cold Spring Harbor group through a quantitative correlation of the different experimental approaches. It is intended to report here on this progress and to consider it in the perspective afforded by a critical review of the present state of knowledge of some aspects of the problem.

1) Various lines of approach based upon different methods of observation of the chromosomal changes are described in Table 1.

distinguishing various types of changes (for example, "point mutations" and "gross chromosomal rearrangements"); *b*) interpreting their different behavior (for example, differential sensitivity to treatments); *c*) testing their independence. One of the chief methods of analysis is the observation of the ratio between the frequencies of different changes as a function of the experimental conditions. For example, the relative frequency of gross chromosomal rearrangements is much larger among X-ray induced than among spontaneous mutations. In the usual radiation experiments, experimental

TABLE 1. APPROACHES TO THE OBSERVATION OF CHROMOSOMAL CHANGES IN DROSOPHILA

Method of observation		Characteristics	
		Favorable	Unfavorable
Cytological analysis	Salivaries	Possibility of revealing by <i>direct observation</i> detailed characteristics of changes (such as the number and location of breaks and the type, frequency and special characteristics of recombination).	Selection by sterility. Inaccuracy of observation of heterochromatin. Amount of labor.
	Mitosis of early cleavages	Independence of sterility.	Limited information due to small size of chromosomes.
Genetical analysis		Possibility of very extensive and accurately scored observations. Flexibility in special investigations.	Inaccurate picture of changes (different changes may have similar genetic behavior). Selection by sterility.
Sterility		Possibility of investigating the selective action of sterility.	Inaccuracy due to large variability and to influence of environment.

Radiations, or any other mutation producing agent, can be used to investigate two main questions: 1) the qualitative analysis of the changes, and 2) the quantitative correlation between the changes and the producing agent. Because of the fruitfulness of initial experiments the attention and activity of investigators has been attracted toward the second of these questions.

Qualitative analysis of the changes aims at: *a*)

conditions are mainly dependent upon the quality of radiation, the quantity of radiation and secondary treatments and biological conditions. The most important characteristics of the different radiations are summarized in Table 2.

Other conditions which have been considered are: relations between the time distribution of irradiation and the biological cycle, temperature at various stages of the experiment, secondary irradiation

TABLE 2. CHARACTERISTICS OF VARIOUS TYPES OF RADIATION

Type of radiation	Distribution of action in space	Magnitude of single energy quanta delivered by radiation to matter.
Ionizing radiations	X-rays and Γ -rays	Variable at random over a wide range; $\approx 30\text{eV}$ on the average.
	Heavy particles	Concentrated along the tracks of particles.
Ultraviolet radiation	Selective absorption according to local properties of matter.	A few eV, corresponding to the weakest component of X-ray action.

with infra-red (see Kaufmann, 1941), the stage of maturation of the germ cells, etc.

A quantitative correlation can be established between the frequency of any class of changes and the physical conditions of the experiment (time, temperature and dosage of irradiation). This correlation is significant in itself only if the class of changes considered is independent of other changes (for example, since the "point mutations" are correlated to the chromosomal rearrangements, the dose dependence of the frequency of point mutations cannot be significant by itself but must be discussed only in connection with the chromosomal rearrangements). The observed quantitative correlations afford an insight into the origin of the changes through interpretations based on particular models of production of the changes.

For instance, Timofeeff and collaborators (1935) assumed that the correlation between the rate of spontaneous mutations and the temperature is essentially the same as that of physico-chemical reactions fulfilling Van t'Hoff's rule. On this basis they were able to measure the energy required for the activation of "point mutations."

The interpretation of the well known dose-frequency correlations has usually been based on the system of somewhat specific assumptions known as "target hypothesis" or "hit theory." A broad formulation of this theory assumes that:

a) The frequency of certain biological "single events" is proportional to the dosage of irradiation, regardless of the connection between the irradiation and the "events"; b) the frequency of biological processes depending on many "events" (e.g. many chromosome breaks) is determined firstly by the random independent occurrence of all the required "single events" and then by their combined action.

In accordance with this theory, the view has been widely accepted that "point mutations" must be considered as "single events" and workers have striven to show that each break involved in gross chromosomal rearrangements should also be considered as a "single event." Attempts have been made, moreover, to correlate the frequency of "single events" per unit dose of radiation with the existence and the size of a "sensitive region" within which radiation or other agents must act in order to produce the "event."

Although the investigation of quantitative correlations appeared to be very successful, it is at present thought possible that this approach has run too far ahead of the preliminary qualitative analysis. Cases in point will be mentioned further on. For example, it is now apparent that the cytologically detectable chromosomal rearrangements consist of many components, so that one should not have expected their frequency to increase proportionally to an integral power of the dosage.

2) This section summarizes the results obtained by the use of single methods of observation sepa-

ately. *The analysis of random salivary glands* in the F_1 of irradiated males has been carried out by Catcheside (1938) and, on a larger scale, by Bauer, Demerec and Kaufmann (1938) and by Bauer (1939). Bauer (1939) gave a very detailed discussion of most of the material available. Although he successfully tested the homogeneity of his results, further experience indicates that it is doubtful whether one can reliably assume that random sampling represents the main cause of variability. The results of Catcheside and Bauer do not agree with each other; those considered by Bauer, which are more extensive, will chiefly be discussed in this paper.

All observed chromosome breaks, regardless of grouping into particular rearrangements, appear to be fairly uniformly distributed on the average among all chromosome sections of equal mitotic length; a small excess of breaks is, however, found in the distal sections. The distribution of the breaks involved in particular rearrangements appears also to be fairly random, but with one important exception: in the case of two-break rearrangements the frequency of inversions bears to that of translocations a ratio approximately twice larger than random, i.e., $\approx 1:2$ instead of $1:4$ (Bauer has attempted to explain this peculiar phenomenon assuming that, in the two-break case only, any large chromosome arm may come into interaction with only two out of the remaining four arms, as well as with itself.)

A rearrangement involving n breaks with such a recombination that the broken ends undergo a cyclic permutation is usually called an n -break contact. Chromosomal changes occur involving many apparently independent contacts: for instance, the formula " $2+2+3$ " represents a rearrangement including three contacts which involve respectively two, two and three breaks. Such a change might be considered, for example: a) as a random recombination of seven breaks which by chance fall apart into three separate cyclic groups, or b) as the random coincidence of three independent changes in a single individual. The first interpretation is closer to Stadler's model (breakage first), the second to Serebrovsky's "contact hypothesis." It is possible to calculate the theoretical values of the relative frequency of various types of changes on the basis of either a) or b); the mathematical consequences of either assumption are not consistent with each other (although this is not apparent in Bauer's analysis). The experimentally determined ratio between the frequencies of " $2+2$ " and " 4 " changes is so large that it cannot be brought into agreement with assumption a). According to b) the relative frequencies of salivary glands involving 0, 1, 2, 3 . . . contacts should fit a Poisson distribution; a deviation of the experimental results from such a distribution appears to be significant (if a sufficiently stringent statistical test is applied). Bauer has attempted to work out a scheme of "limited random recombination" so as to fit all the existing data. His

conclusion amounts to a recognition that the ratio between the frequencies of two- and three-break contacts ($\approx 5:1$) is so small that it is not consistent with the "contact hypothesis." It may also be pointed out that contacts involving odd numbers of breaks seem to be on the whole less frequent than even number contacts.

esses since they arise from two initially independent breaks, while the frequency of independent breaks is proportional to the dosage. Consequently, the frequency of the rearrangements should increase proportionally to the square of the dosage when the dosage is low (as long as it remains negligible as compared to 100%). The increase should be slower

TABLE III. THE RELATIVE FREQUENCY OF VARIOUS TYPES OF REARRANGEMENTS

Dose	2	2+2	3	4	2+3	5	6	2+2+2	2+2+3	2+4 3+3	Other	Total
1000	80% 12 (9.1)	— — (1.8)	13.3% 2 (1.8)	6.7% 1 (0.7)	— — (0.5)	— — (0.1)	— — (0.1)	— — (0.1)	— — (0.1)	— — (0.4)	— — (0.4)	100% 15
2000	73.4% 47 (38.7)	12.5% 8 (7.6)	12.5% 8 (7.7)	— — (2.7)	1.6% 1 (2.0)	— — (0.3)	— — (0.4)	— — (0.6)	— — (0.5)	— — (1.6)	— — (1.7)	100% 64
3000	66.0% 74 (67.8)	11.6% 13 (13.2)	12.5% 14 (13.4)	5.4% 6 (5.0)	2.7% 3 (3.5)	— — (0.6)	— — (0.7)	— — (1.1)	— — (1.9)	1.8% 2 (2.8)	— — (2.9)	100% 112
4000	59.6% 86 (87.1)	14.6% 21 (17.1)	11.8% 17 (17.3)	4.2% 6 (6.4)	2.1% 3 (4.5)	— — (0.7)	0.7% 1 (1.0)	— — (1.4)	2.1% 3 (1.2)	2.8% 4 (3.6)	2.1% 3 (3.8)	100% 144
5000	54.5% 149 (166.3)	11.0% 30 (32.2)	11.5% 32 (32.8)	5.1% 14 (12.2)	6.4% 12 (8.5)	1.1% 3 (1.3)	1.1% 3 (1.8)	2.2% 6 (2.7)	0.7% 2 (2.2)	3.2% 9 (6.7)	4.8% 13 (7.2)	100% 273
Total	60.5% 368	11.9% 72	12.0% 73	4.4% 27	3.1% 19	0.5% 3	0.6% 4	1.0% 6	0.8% 5	2.5% 15	2.6% 16	100% 608

The frequencies of the changes of different types occurring at various X-ray dosages are shown, with their percentual value, in the form of a contingency table (Tables 3 and 4). The "expected" frequencies between brackets have been computed under the assumption that the relative frequencies do not depend on the dosage. Although this assumption has no simple theoretical foundation and although a systematic deviation from the "expectation" is apparent (and is emphasized in Table IV), the fit is rather close in the first columns. The relative frequency of the simplest types of changes, "2," "3," "2 + 2" and "4," is thus nearly constant at all dosages. (The preliminary results of Muller, 1940, with genetic methods on the ratio of changes involving two and three breaks point, however, in the opposite direction.) This result is in definite disagreement with the simple hypothesis that the initial number of breaks produced by radiation is proportional to the dosage. The fact that the sterility selection operates more heavily on complex changes does not bear on this argument.

The dependence of the total frequency of cytologically detectable chromosome rearrangements on the X-ray dosage has chiefly been discussed on the basis of the following assumptions: a) that it is sufficient to consider simple two-break changes as representative of all rearrangements; b) that such changes must be considered as "two event" proc-

at higher dosage following a "two-hit" dose-action curve (so as never to exceed 100%). On the other hand, since many contacts can be produced by chance in the same individual sperm, the total frequency of contacts is not bound to remain smaller

TABLE 4

Dosage \ Type	2 2+2 3 2+3 4	Others	Total
1000, 2000, 3000	19.0% 189 (176)	1.0% 2 (15)	100% 191
4000, 5000	88.7% 370 (383)	11.3% 47 (34)	100% 417
Total	91.9% 559	8.1% 49	100% 608

than 100 percent and might under some circumstances be proportional to the square of the dosage at all dosages. Both of these expectations agree reasonably well with the experimental results.

It is doubtful, however, whether these successful tests are very significant. First, the frequency of

changes other than single two-break contacts is not negligible since it approaches 50 percent at 5000 r. Secondly, the simple hypothesis that the initial frequency of breaks is proportional to the dosage leads one to expect a much more rapid increase in frequency of complex changes than actually found (see above). On the whole, the theoretical interpretation of the dose dependence of the frequency of chromosomal rearrangements is still unsatisfactory.

The analysis of mitotic chromosomes in random embryos of the F_1 of irradiated males has not yet been carried to a quantitative stage. Complex chromosomal rearrangements have already been detected (Sonnenblick, 1940). A quantitative investigation would be valuable because its results would be free of selection due to sterility.

The genetical method of analysis suggests a preliminary classification of the changes into "point mutations" and "chromosomal rearrangements." The "point mutations" appear as changes of localized hereditary factors and can be detected through their morphological effects (phenotypical changes) or through the suppression of all individuals homozygous for the changed factor (recessive lethals). Chromosomal rearrangements are detected as changes of linkage or as inducing hyper- or hypoploidy (duplications or deficiencies) in a series of genetic loci.

Very close connections have been found to exist, however, between point mutations and chromosomal rearrangements, so that a clear-cut classification is not always possible. Coincidence of one break involved in a chromosomal rearrangement with the location of a sex-linked lethal point mutation has been found to be much more frequent than random (Oliver, 1932; Sacharov, 1936; Demerec, 1937). The quantitative data of different authors on this subject agree only as an order of magnitude and are probably affected by the limited efficiency of the genetic methods of detecting chromosomal rearrangements. Evidence that many "point mutations" must be considered as "minute chromosomal rearrangements," chiefly losses of small pieces of chromosome, is given: a) By the frequent occurrence of the genetical inactivation (deficiency) of a whole series of factors located close together along a chromosome, and: b) By the artificial production of short deficiencies simulating point mutations through suitably superimposed chromosomal rearrangements. Furthermore, the "position effect" shows that a change of the linear order of factors may affect their activity.

The effect of radiation on the relative frequency of various types of changes recommends their classification as "gross" or "minute" changes according to whether they do or do not involve large size chromosomal rearrangements (thus minute rearrangements would be pooled together with the "point mutations"). "Gross" changes are exceedingly infrequent not only among spontaneous changes but also among those which are induced by ultraviolet rays

(Muller and Mackenzie, 1940) and by other agents than radiations. It seems to be a characteristic of the ionizing radiations (distributing large size energy quanta) to enhance the frequency of gross changes. The relative frequency of changes affecting one or both chromatid strands within a chromosome, i.e. the "mosaics," has not yet been investigated extensively in *Drosophila* (see, however, Kaufmann, 1941).

It has been known for a long time that the frequency of "minute changes" is proportional to the X-ray dosage when the dosage is low, whereas the frequency of "gross changes" increases much more rapidly with increasing dosage. According to the usual interpretation, "minute changes" should then be considered as "single events," "gross changes" as "many event" processes. Therefore the frequency of sex-linked recessive lethals which are a mixture of "minute" and "gross" changes should, at least at high dosage, increase more than proportionally to the dosage. The experiments do not fit this expectation. Timofeeff (1939) attempted to explain this disagreement by showing that even at high dosage the percentage of gross changes among the sex-linked lethals is so small that it could not affect appreciably the behavior of the whole group. His estimate of the quantitative significance of the gross changes is, however, probably too low, since it disagrees with the direct measurements of other authors (see, e.g., Demerec, 1937) and since his measurement of the frequency of gross changes affecting the X-chromosome does not agree with the information from cytological data (see below).

Genetical methods have recently been applied to investigate certain types of "gross changes" which should depend on a "single event" (i.e. one chromosome break). Pontecorvo (1941) has succeeded in demonstrating losses of the Y-chromosome which behave as "single events," whereas Bishop (1941) has found only a small number of gross "terminal deletions" (losses of large terminal fraction of the X-chromosome).

The sterility method of analysis consists mainly of investigating the ratio of the number of adult flies to the number of eggs laid in the offspring of treated males. This ratio is strongly reduced by an X-ray treatment of a few thousand r. Since the X-rayed sperms contain little but chromosomal material, the sterility is generally attributed to chromosomal changes. Repeated measurements under identical conditions show a large variability which has not yet been brought under control. This prevents the experiments from being carried out with a high degree of accuracy.

The relative viability of males and females can be measured by this method and should give information on the relative import of changes in the Y- and X-chromosome. Gowen and Gay (1933) have reported a decrease of the ratio of females/males approximately proportional to the dosage of X-rays. No such systematic effect has been found in recent experiments by Fano and Demerec (1941).

The induced sterility seems to be proportional to the X-ray dosage at low dosage, as if it were due to a "single event" process. A semi-logarithmic plot of the frequency of survival at high dosage seems to fit a straight line, which is again in agreement with the "single event" picture. Although Gowen and Gay (1933) have reported that the semilogarithmic plot fits a straight line at all dosages, more recent results seem to show the existence of a definite curvature at intermediate dosage (Fano and Demerec, 1941).

3) *Cooperative application of cytological and genetic methods of observation* has taken place over a long period through the cytological analysis of the salivary gland chromosomes of stocks carrying specific genetically detected phenotypical changes. A large amount of data of this type has been collected by Demerec and collaborators and has been kindly made available for this discussion. The qualitative analysis shows that phenotypical changes can be classified into three main types according to whether they are connected with: *a*) no detectable chromosomal rearrangement in the proximity of the changed locus, *b*) loss (deficiency) of a number of chromosome bands including the one connected with the changed locus, *c*) a gross chromosomal rearrangement involving one break adjacent to the changed locus.

The relative frequency of these various types depends on the locus which is considered. Type *a*) represents the closest approximation to the genetic concept of a "point mutation." Most of the changes of type *b*) involve the deficiency of only a few bands, but a number of them involve rather large deficiencies (the upper limit is set by the dominant lethal effect due to the collective action of the deficiency for too many loci. Type *c*) can be further classified according to whether the chromosomal rearrangement has transferred the changed locus to an euchromatic or to a heterochromatic neighborhood. In the first case the break is always very close to the changed locus; but in the second case loci can be affected up to about 15 bands from the break; this last effect is usually accompanied by "mottling."

The next question is whether and how the deficiencies are related to the gross chromosomal rearrangements. If they are related, the deficiency of a small part of a chromosome represents a process alternate to the production of an inversion of the same length, as shown in Figure 1. It is thought that large size deletions arise frequently this way, but that they are not detected because of being inviable. Assuming then: 1) that the deficiency of a certain piece of a chromosome is just as probable as the inversion of the same piece, 2) that the occurrence of such a phenomenon does not depend, on the average, on the particular group of chromosome bands which is involved (this is based on the distribution of breaks observed in salivary glands chosen at random), it has been possible to calculate the expected frequency of the loss of any particular

group of bands on the basis of cytological data compiled by Bauer (Demerec and Fano, 1941). Comparison with the experimental data on the Notch locus has then shown that the changes of type *b*) involving the deficiency of more than about 15 bands can be accounted for as being actually "gross chromosomal rearrangements" which by chance involve only a rather small piece of a chromosome. The shorter deficiencies, on the contrary, are so frequent that they should have a different origin, i.e., they probably are of the nature of "minute rearrangements." To show this, one should prove further that the relative frequency of large and small de-

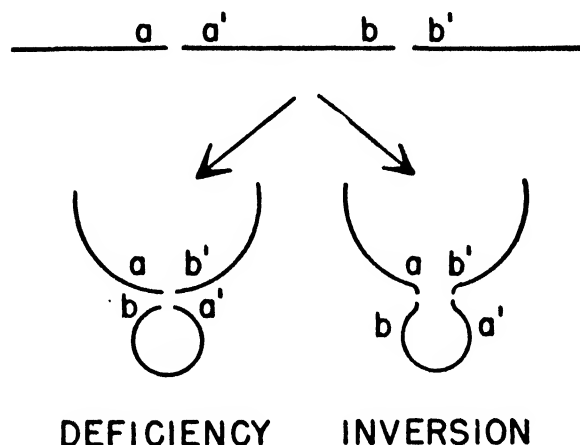


FIG. 1—Diagram indicating symmetry in the production of deficiencies and inversions.

ficiencies depends on the dosage as does the relative frequency of "gross" and "minute" rearrangements. A favorable answer can be expected from experiments in progress, since no deficiency larger than 13 bands has been found among 16 spontaneous changes of Notch. This evidence suggests that short deficiencies do not originate as "gross rearrangements," since these are practically unknown among spontaneous changes.

The same quantitative correlation which has been applied to the investigation of the Notch deficiencies can be applied to various other questions. First, one can test the initial assumption that inversions and deletions are equally probable. Genetical methods afford a measurement of the frequency of the deletions covering nearly the whole X-chromosome, extending from any point between two loci on the tip to any other point between two loci on the base of the chromosome (these deletions are viable when combined with attached X) (Bishop, 1941). The frequency of the inversions covering the same interval have been measured cytologically (Kaufmann, unpublished). Both frequencies are found to coincide within the limits of accuracy of the method (Bishop, 1941).

Secondly, one may evaluate the efficiency of chromosome breaks in producing genetic changes in

the adjacent loci. A genetic experiment by Demerec (1937) showed that 5 out of 10 random X-chromosome rearrangements induced by 2500 r X-rays were connected with lethals. Cytological analysis of random salivary glands shows that the frequency of breaks induced by 3000 r X-rays in the euchromatin of X is about 8 percent (this is the product of the frequency of all breaks at 3000 r, i.e. 49 percent, and the fraction of breaks within the euchromatin of X, i.e. 325/2010; data from Bauer, 1939). On the other hand the frequency of X-chromosome lethals at the same dosage is also approximately 8 percent, out of which a fraction of the order of 30 percent, i.e. ≈ 2.5 percent of the total, is connected with chromosomal aberrations. Thus the efficiency of breaks in producing lethals in X should be, on the average, of the order of 30 percent (i.e. probably larger than 10% and smaller than 100%).

Another question concerns the sensitivity of particular loci to the occurrence of adjacent breaks. The frequency of breaks in either position immediately adjacent to the Notch band (i.e. 3C5-6/3C7 or 3C7/3C8) is approximately $(2/645) \times 8\% \approx 2.5/10,000$. This frequency must be further reduced by approximately 4/5 if only the recombination with euchromatin is considered; this gives $\approx 1/5000$. Experimentally, 11 Notch changes connected with such a break have been recorded out of an estimated total of 740,000 gametes investigated at 2500-3000 r. Thus the sensitivity of the Notch locus appears to be of the order of 10 percent. In the *cut* locus 11 changes of the same type have been recorded out of 100,000 estimated gametes, thus bringing the sensitivity rather close to 100 percent. Whether the difference is actually due to different sensitivity of the loci or to local variations of the frequency of breaks, is not known. (The frequency of breaks in 7B and 7C is particularly large, according to unpublished data of Kaufmann.) A few changes have been recorded, in which the break is not immediately adjacent to the band connected with the locus; thus there appears to be a "sensitive region" which is somewhat larger than one band and especially larger for *cut* than for *Notch*.

The frequency of recombination with heterochromatin is about four times smaller than with euchromatin; on the other hand the action of a break has a range of 16-odd bands instead of approximately a single one. Hence the expected frequency of heterochromatin effects should be about $16/4 = 4$ times as large as the frequency of euchromatin effects. Experimentally, in the case of Notch, this ratio is approximately 2-3.

The frequency of chromosomal rearrangements in the euchromatin of X at 3000 r measurable by genetic methods, is smaller than the total frequency of breaks ($\approx 8\%$), not only on account of the random coincidence of more than one break but also because inversions involving more than one break in the same chromosome are more frequent than

random. These factors may reduce the frequency of the rearrangements in the euchromatin of X to 6-7 percent. This value is, however, still about three times larger than the value of 2.14 percent obtained by Timofeeff (1939). This affects Timofeeff's conclusions (see above) and shows that the genetic methods of detection may be somewhat inefficient.

In conclusion, phenotypical changes should be classified at present into four instead of three main groups, involving: *a*) no cytologically detectable change, *b*) a short deficiency covering the changed locus (minute rearrangement), *b'*) a large deficiency covering the changed locus (actually a gross rearrangement), *c*) a gross chromosomal aberration with a break adjacent to the changed locus.

Furthermore, changes analogous to *b*) and *b'*) but which do not cover the changed locus may affect it through an adjacent break like changes of the type *c*). Types *b'*) and *c*) are not found among spontaneous changes. Short deficiencies might originate the same way as the individual breaks involved in gross rearrangements; this might be tested by investigating through salivary gland chromosome analysis whether an appreciable fraction of random breaks is associated with a short deficiency. It has been doubted whether the difference between types *a*) and *b*) is a real one or whether the distinction is merely set by the resolving power of the methods of observation. In the case of Notch, the changes of type *a*) are about as frequent as single band deficiencies of type *b*).

The correlation between the sterility effects and the cytological and genetical methods is a means of investigating two main questions: *a*) whether and how sterility effects can be traced back to chromosomal changes, *b*) whether and how sterility effects act selectively on those chromosomal changes which come within our range of observations.

Question *a*) must be considered first. It is doubtful at present whether there are "point mutations" (i.e. changes not associated with chromosomal rearrangements) which act as "dominant lethals." In fact, there is no indication of the existence of changes occurring within a locus which can affect an individual more strongly than the actual loss (deficiency) of a small piece of chromosome covering the locus. A large number of deficiencies of moderate size is known, which together cover an appreciable fraction of all loci, and no one of them acts more strongly than a recessive lethal. This makes it also improbable that dominant lethals can be accounted for by short deficiencies (minute rearrangements). Therefore it is thought that dominant lethal effects should arise mainly from deficiencies covering a large number of loci, i.e. gross chromosomal rearrangements involving deletions or the occurrence of dicentric or acentric fragments. The question is then whether the expected frequency of such phenomena, based on the present knowledge, is sufficient to account quantitatively for the observed sterility effects.

Calculations to this end were made by Catcheside (1938) and later by Bauer (1939); further unpublished ones are referred to by Muller (1940) and Pontecorvo (1941). The main difficulty is that one must rely on doubtful assumptions. The equiprobability of inversions and deletions, however, which has been considered above, can be generalized to a broad formulation which is most acceptable as a fundamental assumption. This formulation is: Any particular broken end has the same a priori probability of joining with either one of the two partner broken ends arising from a particular break. Thus any two rearrangements which differ from each other only through the exchange of a pair of partner ends should be equally probable. It can furthermore be assumed, according to the cytological data, that the breaks involved in any particular type of rearrangement are distributed approximately at random along (but not among) the chromosomes. On this basis it is possible to calculate the relative frequency of viable and inviable rearrangements of any possible "type." For this purpose a "type" of rearrangement is characterized by the number of contacts and by the number of breaks involved in each contact together with their distribution among different chromosome arms. Consider, for instance, the "type" of rearrangement defined as: "2+2 with each contact in a single chromosome arm," that is, consisting of two independent inversions or (gross) deletions. Since any one of the contacts has the same chance of being an inversion or a deletion, the odds are 3 to 1 that at least one of the two contacts is a deletion so that the change is inviable. Some information on the frequency of the viable changes of this type can be obtained from Bauer's material: There are 13 "2+2" changes among 595 salivaries of the F_1 of males X-rayed with 3000 r and among a total of 29 "2+2" changes there are 4 pairs of inversions in different chromosomes. Thus the frequency of the viable changes of the "type" con-

sidered here is $\frac{4}{29} \frac{13}{595} = 0.003$, and hence the ex-

pected frequency of the inviable ones is 0.009. This computation should be repeated for all types of changes. This cannot be done very well, however, since the cytological data are not yet available in sufficient amount and detail to yield the required frequency of all types of viable changes.

To overcome this lack of knowledge previous investigators have attempted to determine the frequency of different types of changes on a theoretical basis, assuming e.g. a random recombination of breaks or a random distribution of the breaks among chromosomes. It is clear now (see above) that no such assumption is reliable.

Thus only a few rough considerations can be made at present. The frequency of the sterility effects induced by X-rays in sperms is definitely larger than that of the cytologically detected rearrangements; their ratio is of the order of magnitude of

ten throughout the usual range of the dosage. It is recalled that, whenever gross rearrangements are assumed to be the cause of sterility effects, the frequency of viable cytological changes per treated sperm must be taken as a basis of comparison, instead of the frequency per observed salivary gland. If, e.g., 100 sperms are treated with 3000 r and 50 percent sterility is thus induced so as to cut down to 50 the number of the F_1 larvae which can be investigated cytologically, and if finally among these 50 there are 10 gross changes (i.e. 20%), the inviable changes appear to be more frequent than the viable ones in the ratio $50/10 = 5$ (and *not* $50\%/20\% = 2.5$). If, on the contrary, the sterility effects were independent of the chromosome rearrangements, the larvae which are investigated would show a fair sample of the rearrangements induced in the sperms, so that the frequency of gross viable changes would be actually 20 percent instead of 10 percent.

Theoretically, assuming merely that the frequency of different recombinations is independent of the exchange of partner ends, it is well known that viable and inviable rearrangements should be equally frequent among all the simple two-break changes. On the other hand, inviable rearrangements are more frequent in the case of complex changes. The share of complex changes which is found by Bauer at 5000 r does not account, however, for a ratio much larger than four. The quantitative data indicate that gross rearrangements analogous to those detected cytologically can only account for a fraction of the sterility effects.

At low dosage, 1000 r, the sterility effect amounts to approximately 15 percent, whereas the frequency of the detected viable changes is way below 3 percent according to Bauer (Catcheside's result was, however, of the order of 10%). It seems unlikely that the occurrence of complex changes at low dosage can account for such a ratio larger than 5:1. Moreover this ratio becomes probably still larger at a lower dosage, since the sterility effect seems to be proportional to the dosage, i.e. to behave differently from the gross rearrangements.

At high dosage the frequency of the observed viable rearrangements per treated sperm decreases after having reached a maximum at 3000-4000 r and hence it becomes less and less important as compared with the sterility effects. This decrease means that either the chromosome rearrangements become highly and increasingly complex, or else that some other independent effect is mainly responsible for the sterility.

These considerations, together with the exponential decay of the fertility of sperms at high dosage, suggest that some "single event" process contributes mainly to the sterility effects. The "single break" chromosome losses, due to fusion of sister chromatids, which have been investigated by Muller (1940) and Pontecorvo (1941), do not seem to be important enough, since the frequency of the loss of a particular chromosome is only about 1 percent at

4000 r. Minute "single event" deficiencies should not be large enough to act as dominant lethals; moreover their frequency is also probably too small: The frequency of "minute" Notch deficiencies at 3000 r is of the order of $22/740,000 \approx 3 \cdot 10^{-5}$; when this is multiplied by the number of independent locations in the X-chromosome (a few hundred), it gives only about 1 percent. One might finally think that gross terminal deletions occur frequently as a consequence of a single break and that they are nearly always lethal, by their own nature of involving an unsaturated broken end. Failure to detect a large number of gross terminal deletions in *Drosophila* by suitable genetic set-ups (or to detect large numbers of minute terminal deficiencies) indicates that if such changes occur they must have a particular lethal action, besides that of being deficient for a large number of loci (see also Bishop, 1941). A minimum estimate of the frequency of the occurrence of a single break can be given, if one assumes that the occurrence of an n -break rearrangement is bound to the random coincidence of n independent breaks. This estimate exceeds the frequency of the sterility effects, at low dosage.

It is thus seen that the present state of knowledge in this field is rather unsatisfactory. Besides gathering more accurate data through the usual analysis of salivary gland chromosomes and through fertility measurements, one might achieve some important progress through the quantitative observation of gross chromosomal rearrangements in young embryos so as to get direct, though less detailed, information on the inviable rearrangements. Furthermore direct investigation of certain, usually inviable, types of rearrangements through the use of hyperploids has been already started, especially by Muller and Pontecorvo (1940).

Relatively little attention has been paid hitherto to the question of whether and in what manner sterility effects act selectively on the changes which are observed either cytologically or genetically. The usual assumption that the observed changes do represent a fair sample of the changes induced by radiation in the sperms of the parent male implies that the sterility effects are fully independent of the observed changes. This assumption is not strictly correct. Considering, for instance, all the observed recessive lethals, they certainly involve gross viable chromosomal rearrangements which are not independent of the inviable rearrangements, while these finally represent a fraction of the sterility effects. If, on the other hand, the sterility of sperms could be accounted for by inviable chromosomal rearrangements, selection would certainly affect the observed changes, but could also be taken into account quantitatively in the discussion of the experiments.

Thus the present lack of knowledge on the nature of the sterility effects bars even a rough estimate of the import of selection. It is not impossible that most of the sterility is due to a still unknown process independent of all other changes, so that neglecting

the selection represents, after all, a fair approximation.

Throughout the discussion developed above the sterility induced in sperms has been considered as an "all or none" effect. If, on the contrary, "semi-dominant lethals" should come into consideration, such that sperms carrying a certain recessive character are not fully sterile but their average fertility is below normal, the discussion would be still more difficult.

All these considerations represent approximately the present day line of advance, as it is seen in the Cold Spring Harbor Laboratory; an advance which has not yet yielded a clear picture of the phenomena under investigation. If the phenomena appear now to be more complex, perhaps, than it had been hitherto realized, this means that the advance has progressed just beyond its preliminary stage. All this work has been the object of continuous discussions with various workers, especially with Dr. M. Demerec and Dr. B. P. Kaufmann, whose kind help and advice I gratefully acknowledge.

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DISCUSSION

MULLER: Are all dominant lethals necessarily chromosomal rearrangements? Of course, point dominant lethals could not be detected as such although their possibility is shown, among other things, by such cases as Patterson's so-called "viability gene."

M. BISHOP: This sometimes comes through as an extreme Minute.

SCHULTZ: The question of the point localization of extremely inviable phenotypes, such as dominant lethals, can be analyzed by the study of deficiencies. In his studies, for example, Bridges found that the individual effects were sharply localized, and that the effect of the deficiency as a whole was the sum of such individual effects. Thus it is not excluded that point dominant lethals occur.

FANO: This report is that of the general feeling of the Cold Spring Harbor group, not my own

exclusively, and the essential view is that point mutations cannot be responsible for most dominant lethals.

SAX: The distribution of the number of two and four strand exchanges can be tied up with spatial relations; there is evidence for this in *Tradescantia*.

FANO: The *Tradescantia* phenomena produced by Sax gave an effect of opposite direction to that of *Drosophila*; too few double rearrangements were obtained, while we find too many. In the *Drosophila* material, at 5000 r the results fit a Poisson distribution but at 2000 r they do not, which is very odd.

MULLER: What was the absolute number in the experiments at 2000 r?

FANO: 47 single and 8 double breaks.

MULLER: Have you any suggestions to account for this peculiarity in distribution, supposing it to be real?

FANO: If it were really so, this would mean that radiation only starts the chromosomal rearrangement, but the relative frequency of the different types of rearrangement is determined by the chromosome phenomena themselves independently of the excitation.

SCHULTZ: Might the linear dosage relation in Catcheside's material indicate an intensity effect similar to that found in *Tradescantia* by Sax?

FANO: The effect was not found by Muller.

MULLER: Would heterogeneity of the material from cell to cell account for any of this?

FANO: Kaufmann finds serious heterogeneity at high dosage. But a large number of slides were not examined in previous experiments.

MULLER: I see no evidence that heterogeneity exists, one way or the other, except this of Fano's.

FANO (to Demerec, who passed it to Muller): Are point dominant lethals known?

MULLER: Though cases completely fulfilling this definition could not be detected, one approaching it was a case of mine reported in 1921, where I got three allelic mutations: broad, a broad-lethal recessive, and a third allele, which gave a dominant broad effect when crossed to normal and which acted as a dominant lethal when broad was in the other chromosome.

POUGH: A similar case is the most extreme vestigial allele "no-wing," which is dominant sterile in males, and at least semi-lethal. This is true of the extreme alleles of other multiple allelic series.

DEMEREK: kz/kz^{def} is another combination with dominant lethal effect.

MULLER: Some scutes are in this class also.

SCHULTZ: Are exaggeration effects needed to explain this? Some Minutes are pretty close to being dominant lethals of this type.

DEMEREK: Would you expect a deficiency for a certain locus to give the most extreme effect at that locus, or would you expect that a mutant allele may be more extreme than a deficiency for the same locus?

MULLER: It seems probable that most mutations

are hypomorphs, and these would give no more extreme effects than deficiencies.

DEMEREK: If it is assumed that as a rule a deficiency at a certain locus is more injurious to the organism than any mutation which may occur at the same locus, then it should be possible to detect dominant lethal loci through deficiencies. In our collection of deficiencies we have adequate material to cover almost the whole left half of the X chromosome. In that region there seems to be only one locus which approaches dominant lethal classification, namely, Patterson's viability locus at the tip of the chromosome. However, it is well known that this locus represents an extreme Minute which survives only in most favorable circumstances. This evidence suggests that in *Drosophila*, dominant lethal loci are not numerous and that dominant lethals obtained in X-ray experiments are too frequent to be accounted for by changes in genes.

SCHULTZ: I do not think that the distribution of dominant lethals among the loci in the chromosomes is sufficiently studied as yet to allow generalization.

MULLER: Examination should be made of more known mutants to determine definitely whether the great majority are hypomorphs. If so, this would tend to corroborate Fano's theory.

FANO: Assuming that point dominant lethals represent only extreme types, and that there is a comparable amount of semi-dominant lethals, and assuming further that point dominant lethals represent the bulk of all dominant lethals, one would expect a tremendous number of semi-dominant lethals to be transmitted to the F_1 of rayed males and to show up in the F_2 . This is not observed.

STADLER: Isn't Demerec's argument for the absence of dominant lethals made from the part of the map where they would be least expected? A dominant lethal locus is one where the wild type allele is haplo-insufficient for life. These may be present in the autosomes but absent in the X for evolutionary reasons.

DEMEREK: Data for autosomes are hard to get, but a sufficient number of autosomal deficiencies has been studied to show that if there are point dominant lethals, they would have to be restricted to certain regions and not distributed at random.

STADLER: How numerous would they have to be to be responsible for the sterility effects?

FANO: They would have to be exceedingly numerous.

MULLER: Analysis of triploid offspring of irradiated males would give some evidence on this question, as it would allow some offspring to survive carrying genes which in a diploid would cause inviability. It would be better still to use duplications for sections, to avoid using complete triploids. In an experiment of the latter type, carried through by Alikhanian, one dominant lethal (involving loss of the "bulb") was found among about 22 lethals produced in the left end of X.

A THEORY OF AUTOCATALYTIC SYNTHESIS OF POLYPEPTIDES AND ITS APPLICATION TO THE PROBLEM OF CHROMOSOME REPRODUCTION

M. DELBRÜCK

I. AUTOCATALYTIC SYNTHESIS

A discussion of normal chromosome behavior and of its disturbances by irradiation is hampered by our lack of knowledge of the chemical reactions in which the gene is involved. Of these reactions we presume there are many but we are certain of only one, the reproduction of the gene, which normally occurs once for every gene in every cycle of the cell. No chemical theory of this reproduction has yet been proposed, let alone proven, and the process of reproduction has not become an essential part of the interpretation of normal and abnormal chromosome behavior. One finds terms like "the moment of reproduction," "the time of split," "the time of effective split" in the literature, where they are used to explain the cyclic variations in the reactions of chromosomes to external agents. While such a procedure seems justified as a heuristic method it has led to difficulties and contradictions, inasmuch as the "time of effective split" turns out to be very different depending on the agent under consideration. The reason for this must lie in the fact that the process of reproduction and splitting is not confined to a definite phase of the cell cycle but stretches over a large part of it and various agents will selectively interfere with one or another phase of it.

Under these circumstances it seemed desirable to examine the essential difficulties of a chemical theory of reproduction in order to get a better understanding of the requirements which such a theory will have to meet.

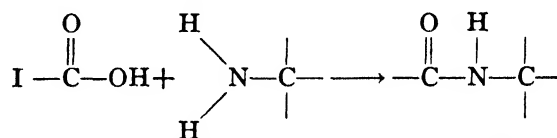
There are three main difficulties. The first concerns the source of energy needed for the synthesis of polypeptides; the second concerns the autocatalytic nature of the reproduction; the third concerns the fact that in the normal cell the reproduction stops after the production of just one replica.

In this paper we propose a scheme of synthesis which illustrates the manner in which these difficulties may possibly be overcome.

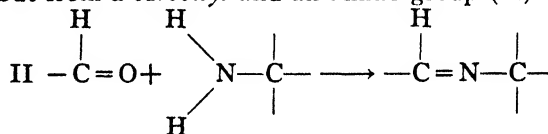
1) Synthesis of polypeptides from amino acids requires energy; it must therefore be coupled with some process yielding free energy. Such coupling of different reactions has recently been analyzed successfully in the case of phosphorylations and oxidations, where it could be shown how the energy of oxidations is made use of for the synthesis of high energy phosphates (Lipmann, 1941; Kalckar, 1941). The salient point is this: instead of carrying out an oxidation of the substrate which would set free a large amount of energy the oxidation is carried out on the phosphorylated substrate. The energy is

not liberated but instead the phosphate bond is transformed into a high energy (unstable) bond. This high energy phosphate group can then be used to carry out in a spontaneous reaction any desired phosphorylation.

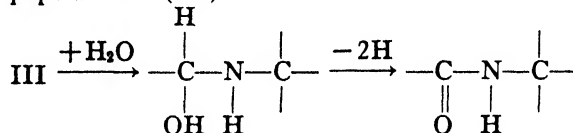
In analogy with this Linderström-Lang (1939) and Kalckar (1951) have suggested that the synthesis of the peptid bond is also coupled with oxidations, which supply the energy. They propose that the peptid bond is not formed directly from a *carboxyl* group and an amino group (I),



but from a *carbonyl* and an amino group (II)

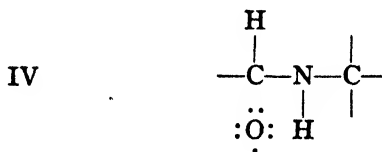


with subsequent oxidation of the imid bond to a peptid bond (III).



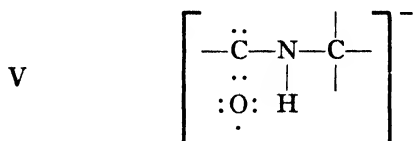
2) The imid bond formation does not require energy and does not require an enzyme. Any specific catalysis of the synthesis could occur only during the subsequent oxidation. It seems to me that a theoretical argument can be advanced for the occurrence here of a catalytic mechanism that would be highly specifically *autocatalytic*.

In order to oxidize the imid compound, two hydrogen atoms have to be taken off. The difficulty of such two step oxidations, from the energy standpoint, lies in the fact that the two steps will be energetically of very different size, because the intermediate compound (IV), having one unpaired electron,

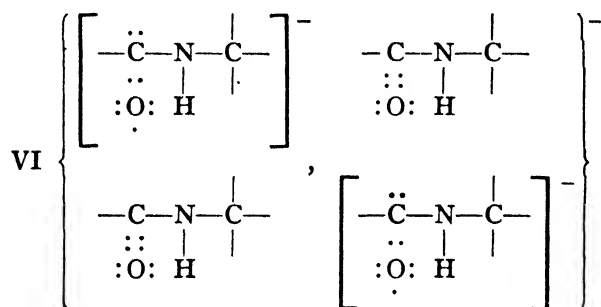


will be a high energy radical. It would therefore require an oxidant of very high redox potential to per-

form this oxidation, which the cell is not likely to possess. The situation would be helped if the energy of the radical, or of its dissociation product (V) were decreased by resonance between states of equal energy, as in the semiquinones of Michaelis (1939).



Clearly a complex between (V) and a molecule containing the peptid bond is suited for this purpose, because the latter differs from the radical by only one electron, and will therefore be able to form a one electron bond with it; (Pauling, 1939) thus reducing the energy of the radical (VI)



This reaction will be specific for two reasons. First, the catalyst must fit sterically with the substrate, in order to permit close contact; second, the energy of the additional electron in the substrate must be close to the energy of the same electron in the corresponding state in the catalyst.

Both these requirements will best be fulfilled by a catalyst with a structure very similar to that of the product of oxidation. The reaction will therefore be autocatalytic and to a high degree specifically so, although the specificity will have its limitations as far as more distant parts in the side chains of the substrate are concerned.

The essential features of this scheme are:

a) It links the synthesis with intermediate metabolism, more specifically with oxido-reduction.

b) It explains autocatalysis not by means of a mysterious attraction between like molecules, but by a short distance interaction, which does not attract the correct substrate, but which reduces its energy of oxidation, thus selecting the correct substrate for oxidation, and thereby also for the synthesis.¹

3) The scheme may also explain why reproduction is normally confined to the synthesis of just one replica. It separates the peptid bond formation into two oxidation steps. The two steps may

well be separated in time, in such a manner that one step is first carried out along the entire length of the chromosome, before the second step is initiated. The intermediate situation would then be a semiquinone double chain, with a negative charge for each prospective peptid bond.

It must be supposed that the two steps of oxidation require different redox conditions, so that each can occur only at a particular phase of the cell cycle, and the first step, the formation of the intermediate complex, cannot be repeated until the second oxidation has restored the original condition.

II. THE FORCES RESPONSIBLE FOR THE CHROMOSOME MOVEMENTS IN MITOSIS AND MEIOSIS

The question arises whether the alternation between the charged intermediate state and the uncharged polypeptide stage can be brought into relation with the periodic variations of the forces between the chromosomes, which can be inferred from the movements of the chromosomes. These forces seem to be in part of an electrostatic nature, and as such must be repulsive between homologous elements of the dividing cell. It would seem that the separation of the daughter chromosomes at the beginning of anaphase of mitosis could be ascribed to the acquisition of a charge by each daughter chromosome at this stage. In our theory that would mean that at this stage the first half of the reproduction process should be completed, and that the daughter chromosomes moving to the poles are in the semiquinone stage, that is, they are microscopically not separable double threads. It should be noted however, that the chromosomes at this stage are easily oxidizable and that such an oxidation may occur during fixation, with subsequent separation of the half chromatids so that the doubleness of the anaphase chromosomes, which has been claimed by many cytologists, may be a fixation artifact.

In meiosis the formation of the double thread is preceded by the pairing of homologous chromosomes. The forces operating in this pairing cannot be long distance forces, for two reasons. First, the two homologues must be assumed to be in equivalent states, therefore, forces arising from electric charges would be repulsive. Second, the forces must be specific for the chemical constitution of each chromomere, and it has not been found possible to construct a physical model for specific attractive long distance forces. I think that the claims of various authors to this effect are not justifiable by accepted physical principles. But it would also appear that the phenomenon of pairing does not demand the assumption of the presence of this type of forces. The pairing between homologues should be interpreted by short distance chemical interactions. It must be initiated by a special mechanism at some homologous places of the chromosomes and then continued along the thread in zipper action.

Various authors have pointed to the similarity of

¹ The idea that autocatalysis of the type here required could be caused by the change in energy, due to a resonance, of an intermediate state, is implicitly contained in a short note by Frank-Kamenetzky (1939, and personal communication).

this pairing phenomenon and the reproduction of the chromosomes. The similarity may be more than superficial. In our scheme it would mean that the pairing is an intimate association of the self reproducing entities within the chromosome coupled with the chemical reduction of each paired pair of peptid bonds, so that between each pair the resonance bond (V) can be formed, which we postulated as an intermediate for the synthesis of new peptid bonds in polypeptide synthesis. The assumption would make the "pairing force" locally specific and normally restricted to pairs, in agreement with the facts. A change in the redox situation of the nucleus would then reverse this reduction and permit both the separation of the homologues and, by addition of new material and further oxidation, the synthesis on each homologue of the semiquinone complex. The two homologues would then show electrostatic repulsion, which is the characteristic situation at diplotene and diakinesis.

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DISCUSSION

METZ: The question of repeats is interesting in relation to the idea of zipper action. Repeats come together in salivary gland chromosomes, although far apart in the chromosome complement, in the same way as homologues. This is the same principle as synapsis.

DELBRÜCK: I think pairing in the salivary chromosomes is not quite comparable to pairing in meiosis, because it is not restricted to pairs.

METZ: Are you thinking in terms of a single molecular chain or a compound one, a number of molecules or a single molecule?

DELBRÜCK: I don't know.

WRINCH: Dr. Delbrück has called attention to the many deep lying difficulties in the chromosome problem, among which he has specially mentioned the fact that synthesis apparently stops with duplication. I would suggest that this difficulty may perhaps be met by the picture of such synthesis as due to the apposition of parallel surfaces.

In connection with the chemical scheme put forward by Dr. Delbrück, I would like to ask whether it is reasonable to indulge in speculations regarding the process of condensation of amino acids in which the nitrogen component plays no role? It is my impression that in a very great variety of ways, e.g. spectroscopically and in numberless organic reactions, it has already been shown that the C=O

group is profoundly affected by the juxtaposition of the nitrogen atom.

DELBRÜCK: Nitrogen has no special role in the scheme as outlined. I should like to emphasize that the scheme even if wholly true will represent only a small part of the whole truth of this matter. For one thing, nucleic acid has not even been mentioned, and it must certainly have an important function. Also, nothing was said about the oxidants concerned. It was not my intention to develop a complete picture, but to point out an implication of the Linderström-Lang's scheme, which seemed hopeful to me.

WRINCH: In his scheme for chromosomal synthesis, Dr. Delbrück has confined his attention to the matter of duplication of such minor parts of the proteins as the CO-NH grouping which he assumes they contain. It is my reading of the biological facts and the facts established by enzyme chemistry that the particular R-groups which are present must also be taken into account. For complete duplication, it is necessary to explain how the same spatial inter-arrangement of specific residues is obtained. On the lines which he suggests, in which he is concerned solely with polar forces, it might be possible to work in R-groups, which are themselves of polar type. Even this would seem to be difficult, but his scheme takes no account of the complement of non-polar side chains which all known proteins contain. A pattern of non-polar R-groups could only impose its pattern on new material if some account be taken also of the geometrical and chemical affinities of groupings capable of exerting only the milder van der Waals forces, which are of course also less well defined stereochemically. I feel his scheme fails in that it cannot account for synthesis involving groupings of this kind. That these groupings are of the first importance in biological specificities in general is shown by a wide variety of physiological phenomena.

DELBRÜCK: The scheme is concerned with the synthesis of the peptid bonds. But the synthesis as outlined is specific for the side chains for two reasons. First, the steric fit must be close, otherwise the resonance energy will be very small. The resonance energy decreases very rapidly with the distance. Second, the extra electron must have closely the same energy in the catalyst and in the substrate molecule. This energy will be influenced by the side chains.

MULLER: Would the molecules come together "front to front" or "front to back"?

DELBRÜCK: The latter, as in a crystal.

CHILD: Do the different substances which make up the genes reach these specific regions by chance diffusion and does the entire gene not act as the auto-catalyst selecting the proper substances, building them together with the release of unused substances and end products?

DELBRÜCK: In all enzymatic reactions in the cell, the enzymes always select the substances on which they can act, reaching them by diffusion.

CHILD: Then we should consider the whole gene as the duplicating mechanism, and not one active spot.

WRINCH: Dr. Delbrück has been directing his study of protein synthesis on the hypothesis that proteins are linear arrays of residues in which each residue is added successively to a chain of ever increasing length. It is my belief that such a postulate is untenable and misleading. In this connection it is of interest that Dr. Delbrück finds it necessary for some purposes to assume that the enzyme (itself presumably protein in character) is capable of holding the constituents of the new structure in position on its surface in some rigid spatial interarrangement. This assumption is of course in full accord with deductions to be drawn from the many immunological, crystallographic, physico-chemical and physiological protein researches, all of which unite in giving a picture of the protein as a highly organized structure in which the skeletal atoms form a definite spatial pattern from definite positions in the surface of which there emerge some or perhaps all of the various R-groups upon which the specificities of individual proteins depend. I suggest therefore that this picture rather than the traditional linear picture may prove to be a more fruitful assumption in the study of chromosomal reproduction.

GREENSTEIN: 1) The hydrated amino compounds would lose water immediately; the equilibrium really lies on the side of dehydration under ordinary circumstances. 2) Semi quiones are generally colored. No protein is colored without prosthetic groups in it.

FANO: Why do you think there is front to back apposition and not mirror-image?

DELBRÜCK: To get closer contact. Also otherwise you would get synthesis of enantiomorphs.

MIRSKY: Delbrück's theory postulates an oxidation-reduction mechanism. What is actually known about the dehydrogenases of the nucleus? In particular, what changes in the enzymatic system of sperm take place in spermatogenesis?

DELBRÜCK: There is no evidence of which I am aware.

SCHULTZ: Some information on respiratory metabolism in the nucleus is available from Brachet's work on the germinal vesicle of the frog egg. He found a very low respiratory activity in this nucleus, which is very active in synthesis.

NEBEL: The evidence of cytologists must have the reactive regions on the surface of the chromosome, which may have a dorsoventral symmetry. You may have bundles of threads in visible structures, the surfaces of which would get the two bundles together.

HUSKINS: I think we are dealing with entirely different orders of magnitude. The cytologist is dealing with strands which are enormously large compared with protein strings. There is obvious evidence that intracellular forces on a larger scale are involved in distance conjugation, orientation to the

poles, etc. Dr. Delbrück is talking about a different scale. How does his theory relate to chromosome movement, and such problems?

DELBRÜCK: It may be that pairing is something entirely different from reproduction. There may be nothing to the relation.

FANO: Speaking of speculations, the difficulty of getting from a single strand to bigger things could be obviated by thinking of the active part of the chromosome as a surface with an indifferent interior. Then the difficulty would arise that reproduction would be front-front and not front-back.

SPIEGELMAN: Dr. Delbrück, your picture of the synthesis imposes rather severe geometrical constraints on the course of the reaction. Were we to depend on chance collisions to satisfy these constraints the reaction would probably be too slow. Assuming one part, i.e. the already existing structure, held rigid, the conditions would still seem rather severe without assuming any direction forces. Have you calculated the rate for such a reaction?

DELBRÜCK: Yes. Diffusion rates can easily account for very fast enzymatic reaction.

WRINCH: I think it very interesting that Dr. Delbrück finds it necessary to assume that the process of enzymatic action requires that the elements involved be held in a rigid orientation.

MULLER: Why should the back of the structure fit its front? If this is not so, the system would not work. Or is the answer to this that only systems which did work in this way could have survived?

DELBRÜCK: We must postulate that they do fit front to back.

HUSKINS: Would this not explain how chromosomes synapse when they come together, but not why they come together?

DELBRÜCK: They come together at one point, then the rest comes together in a zipper effect.

JAKUS: The kitten ovary gives some visual evidence for this. In the zygotene, homologous chromosome loops come together and lie side by side. Then the chromomeres at the ends fuse, and synapsis occurs by what looks like a zipper action.

STERN: Metz' point has not been answered by Delbrück. If two repeats are far away, they do more than zipper together, but come from a distance to synapse with each other.

DELBRÜCK: I think this is due to movement.

DEMEREK: This pairing is not 100 percent.

SCHRADER: Long distance pairing of sex chromosomes also occurs.

METZ: Synapsis of the X chromosome repeat in *Sciara* is close to 100 percent. A lot of chromosome movement is certainly going on, however, in these nuclei, if the chromosome knots mean what we think they do. It seems necessary to consider the chromonema as molecularly multiple in order to account for synapsis. One of the most interesting problems is the accurate longitudinal splitting. How would this work out in a polymolecular gene?

DELBRÜCK: How can we know what a gene looks

like when we do not know what globulin looks like?

FANO: Have you actually evaluated the probability that a substance diffusing reaches the place of autocatalysis at a reasonable rate?

DELBRÜCK: Many calculations have been made of rates of enzymatic reactions; even in the fastest reactions the diffusion rate is not the limiting factor.

HUSKINS: 1) Pairing is not entirely a zipper action; many cases of interlocking and other phenomena show that it starts at many places.

2) Basing this theory, as Delbrück does, on Darlington's points of view, we should then take another Darlington idea, that of secondary pairing, an utterly different phenomenon.

3) If the chromosome molecule is multiple it must be able to split accurately into two parts. Is it possible that there is a regular compensation

mechanism, such as a saturation point, which causes it to build up to a uniform state?

CHILD: There must be some regulatory mechanism, since chromosome healing occurs, such as occurs after irradiation.

METZ: Probably the ordinary conception of healing is wrong. Chromosome rearrangement may not involve breakage, but may, as Belling suggested, be essentially like crossing over—but of a non-homologous type. If the chromonema is not actually broken, healing does not require union of free ends.

SCHULTZ: The fact has been neglected that we know something about the material the chromosome is made of. Both the nucleic acids and the proteins can undergo polymerization and depolymerization. We have here a good model for association and dissociation of chromosomes.

SPONTANEOUS MUTABILITY IN DROSOPHILA

H. H. PLOUGH

When Muller in 1927 first clearly demonstrated that mutations could be induced by X-radiation, I think it was generally believed that the discovery would be most useful in testing evolutionary theories, or as a practical method for producing particular desired mutations, rather than as a precise tool for the analysis of the nature of the gene. Yet in the intervening fifteen year period chiefly as a result of the use of radiation techniques, there has emerged a new and chemically more satisfactory picture of the gene than was held in earlier days. Though the details have been by no means filled in, nevertheless the older crude model of a row of beads attached to a chemically unrelated strand, has now been replaced with a concept which visualizes the genes as definitely placed entities, loosely combined at intervals with an intervening fibrous protein. This refining process, together with the present confusion in the interpretation of chromosome breaks and deficiencies, has led some competent investigators to question the existence of mutations in the older sense at all. Goldschmidt has become so enamoured of the possibilities of position effect that he has allowed himself to be widely quoted as advocating the complete abandonment of the gene concept. "In other words there are no genes, no gene mutations and no wild type allelomorphs" (Goldschmidt, 1938). If this statement were to be taken at its face value, it would mean that recent genetic studies had been the instruments for completely destroying the whole well established foundations of modern genetics, which it has taken so much labor to build. Certainly the majority of genetics investigators, and perhaps Goldschmidt himself, consider that these newer discoveries simply require the re-interpretation of an older, too simple concept of the gene, and that the problem is really one of integration of recent observations with older hard won basic facts. It seems to be my function in this symposium to recall some of these basic facts which have a bearing on the nature of the gene.

In the enthusiasm for the more interesting radiation studies one important matter which has been shifted from the center of interest is that of spontaneous mutability. No species is genetically static in nature, and it is worth emphasizing that from the point of view of organic evolution the constant occurrence of spontaneous mutations is the basis from which natural selection proceeds. Even for the study of the effects of radiation a sound body of data is required on spontaneous mutability. This requires an accurate record of mutation frequency under the varying conditions which occur in nature, and among these variables none is more important than temperature. For the past six or seven years our Am-

herst group of investigators, consisting of Child, Ives and myself have focussed our attention especially on the analysis of the effects of temperature on mutation frequency in *Drosophila melanogaster*. In order to secure effective temperature control, we have built a constant temperature room about 15' \times 9' with walls of double layers of cork surrounding an air space, sealed outside and plastered on the inside. This room is automatically controlled at $15^{\circ} \pm 2^{\circ}$ and 70 percent relative humidity by a Carrier air conditioning system giving continuous air circulation. Within the room we have 12 triple-jacketed incubators of the Plunkett-Bridges type, each with a continuous fan to keep the inside air in circulation. The different incubators are maintained independently at 2.5° intervals above room temperature and in addition a series of five incubator compartments built into a large Frigidaire give temperature intervals below that of the room. Thus we have compartments for simultaneous experiments at all temperatures from 4° to 40°C . The temperatures and the humidity are automatically recorded every 15 minutes on a continuously feeding paper strip by a Leeds and Northrup recorder from nickel resistance thermometers placed in any eight of the compartments. Thus we are certain that temperature and humidity have been continuously maintained during the experiments.

In reviewing the data on spontaneous mutability which have been secured I wish to emphasize that they are the work of Child and Ives, as well as myself, and the views expressed are our composite opinions even though I am responsible for putting them together in this review. In addition I have tried to summarize the work of other investigators who have interested themselves in the problem of spontaneous mutation in *Drosophila*. In general, I believe that the data support current views of the mutation process and make a consistent pattern with the results from radiation experiments. They help to establish the idea of the chemical nature of gene mutations as phenomena separable from breakage and rearrangements in the chromosomal substance between the genes.

METHODS AND EARLIER DATA

If chemical processes are involved in mutation it would be expected that increased temperature should result in a rise in mutation frequency in accordance with the Van't Hoff rule. Muller (1928) made the first critical experiments to test this and found a two to three fold increase in both visible and lethal mutations in chromosomes 1 and 2 with an increase of 6 to 7 degrees in temperature. The number of tests was large and the methods developed were

essentially those which have now come into general use for the investigation of mutation rates, but since the increased frequencies recorded were low when compared with the more striking changes produced by radiation, they were generally lost sight of. In 1929 Goldschmidt and, independently, Jollos (1930) showed that short exposures of *Drosophila* larvae to sub-lethal high temperature (37° for 12-24 hours) gave an increase in the frequency of mutations as compared with controls at constant temperatures. These results were complicated by the difficulty of separating out the numerous transient modifications which appeared, and also, in Jollos's work, by the claim of specific step-by-step mutations with repeated exposures to high temperature shocks. That an increase in mutation frequency of a lower order

for Gottschewsky's evidence, already on record, (1934) that brief exposures to low temperature also increased mutation frequency.

The conflict which this discovery introduced made it apparent that the whole problem must be re-investigated using tests over a range of temperatures, and criteria of mutations in which individual judgments are as little as possible involved. Muller had long since made available the CIB method for the detection of sex-linked lethals, and Timofeff-Ressovsky and Buchmann undertook an extensive investigation to analyze the conflicting results by its use. But the frequency of sex-linked lethals at 25° is so low that an excessively large series of tests are required to get statistically satisfying differences using agents which, like temperature, produce at best changes in frequency of a low order of magnitude. The autosomes are much longer and preliminary tests indicated that they showed a considerably higher mutation frequency than the X chromosome. In our laboratory, therefore, it was decided to test lethal mutation frequency in chromosomes 2 and 3 using a method first indicated by Muller, although our particular scheme was set up by Child. The method is shown in Figure 1 and involves the use of a stock containing dominant markers in each chromosome and a crossover reducer in one member of each pair. By mating to a $+/+$ stock it is possible to isolate one chromosome of each pair which has been exposed to any particular temperature (generation 1), to duplicate each of these chromosomes (generation 2), and then to mate flies with these identical or isogenic chromosomes to each other (generation 3). The absence of either or both the homozygous classes among the offspring in generation 4 indicates the presence of a lethal in either or both chromosomes. Unlike the sex chromosome which is lethal-free in every male, tests of the autosomes must be begun with stocks which have been rendered lethal-free in a preliminary series of matings, since most stocks carry a certain number of accumulated autosomal lethals in heterozygous condition. The autosomal tests are also more laborious in that they require one more generation than those for chromosome 1.

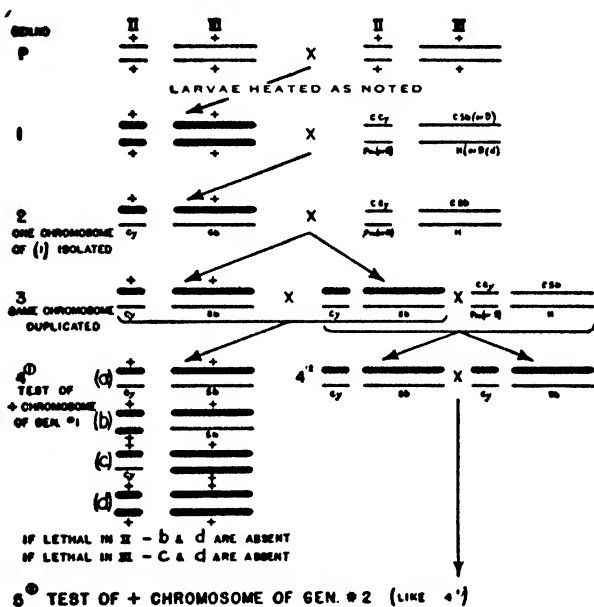


FIG. 1. Scheme for Detecting the Number of Lethal Mutations per Generation in Chromosomes II and III of *Drosophila*

occurs following such shocks was confirmed by a number of investigators, especially Rokitsky, Plough, Grossman and Smith, and further reports by Jollos. In the most extensive report of this series in which the data for each generation were tabulated Plough and Ives (1935) found an increase in visible mutations of over five times in more than 200,000 flies examined. In this study no evidence was found of the specific or step-by-step mutations recorded by Jollos, and this claim seems to have disappeared for lack of confirmatory evidence. Buchmann and Timofeff-Ressovsky (1936) also published an extensive confirmatory series of tests for sex-linked visible mutations and found a two-fold increase. Thus we should have been justified in assuming that the evidence showed that mutation frequency is extremely sensitive to temperature, had it not been

MUTATION FREQUENCY IN THE DIFFERENT CHROMOSOMES

Our first objective was to compare mutation frequency in the different chromosomes of the same Florida #10 stock, the chromosomes of which had previously been rendered lethal-free. The CIB method was used for testing chromosome 1, and the mating scheme of Figure 1 for the two autosomes. The summarized results are shown in Table 1, where it appears that the frequencies of lethal mutations at 25°C are greater in chromosome 2 than 1, and in 3 than 2. If this percentage for chromosome 1 is taken as 1, then that in 2 is 1.60, and in 3 is 2.35. The autosomes are of course larger than the X

chromosome, and Bridges calculated the relative numbers of genes in 1 and 2 as 1 to 1.70. If all genes are on the average equally likely to mutate

TABLE 1. LETHAL FREQUENCIES AT 25°

Chromosome	No. chrom. tested	No. lethals	% lethals
I	916	10	$1.09 \pm .22$
II	1661	29	$1.75 \pm .22$
III	664	17	$2.56 \pm .41$

we should expect a comparison of mutation frequencies to give approximately the difference shown. The third chromosome has only about 10 percent more genes than the second, however, so that its mutation frequency is higher gene for gene, or else certain genes in this chromosome mutate more frequently. It will appear later that there are other

some 2 was given in the following year (Plough and Child, 1937). A complete review of the data was given by myself at the Seventh International Congress of Genetics at Edinburgh in 1939, but due to the difficulties of publication all but an inadequate abstract was omitted from the proceedings. I am, therefore, giving a somewhat more detailed account of the work than would ordinarily be necessary in a review of this kind, including certain results which have become available during the intervening two year period.

The data to show the relation between spontaneous mutability and constant temperature are summarized in Table 2. Timofeff-Ressovsky's frequencies of chromosome 1 lethals appear at the left, and ours for chromosome 2 at the right. Our own data on mutation frequency in chromosome 2 are so few in number that certain of the values are not statistically significant, yet the trend is un-

TABLE 2. THE RELATION OF LETHAL MUTATION AND CONSTANT TEMPERATURE DURING DEVELOPMENT

(Data for chromosome 1 from Timofeff-Ressovsky 1935).
(Data for chromosome 2 from Plough 1939).

Temperature egg-imago	Duration of development	Chromosome 1		Chromosome 2			
(The figures given are: no. chromosomes tested, no. lethals found, percent of lethals with Probable Error.)							
8° (15 days)	30 days			179	0 <table border="1"><tr><td>0.00</td></tr></table>	0.00	
0.00							
14°	22 days	6871	6 <table border="1"><tr><td>0.09 ± .023</td></tr></table>	0.09 ± .023			
0.09 ± .023							
18°	20 days			197	0 <table border="1"><tr><td>0.00</td></tr></table>	0.00	
0.00							
Controls 23°-25°	12-14 days	3708	7 <table border="1"><tr><td>0.19 ± .046</td></tr></table>	0.19 ± .046	979	8 <table border="1"><tr><td>0.82 ± .19</td></tr></table>	0.82 ± .19
0.19 ± .046							
0.82 ± .19							
28°	11 days	6158	20 <table border="1"><tr><td>0.33 ± .048</td></tr></table>	0.33 ± .048	210	2 <table border="1"><tr><td>0.95 ± .45</td></tr></table>	0.95 ± .45
0.33 ± .048							
0.95 ± .45							
31°	9.5 days			72	2 <table border="1"><tr><td>2.77 ± 1.30</td></tr></table>	2.77 ± 1.30	
2.77 ± 1.30							

reasons for believing that mutation frequency is not always proportional to the number of genes under exactly similar environmental conditions.

RELATION BETWEEN MUTATION FREQUENCY AND TEMPERATURE

Both Timofeff-Ressovsky and we have had the same questions to be answered, namely 1) what is the mutability at a series of temperatures held constant during development, and 2) what is the effect of temperature shocks. Timofeff-Ressovsky published his conclusions on the basis of a large series of tests of chromosome 1 lethals in 1935 and 1936, and a preliminary account of ours for chromo-

mistakable and the curve is self consistent. They were all determined for a single Florida #10 stock in the same generation and to add other data would introduce other factors which, as our later work has shown, are certain to modify the mutation rate as much or more than the temperature. In addition at 31° it is especially difficult to get any fertile offspring whatever, and this constitutes the only observation of mutation frequency which has ever been secured at so high a temperature. When it is borne in mind that every one of the tests listed in Table 2 has involved handling a separate mating for three (chromosome 1) or four (chromosome 2) generations, and that the latter also require a pre-

liminary four generation run to get lethal-free chromosomes, I think it may be understood why the data are not more extensive. They are shown as curves in Figure 2. They indicate that mutation frequency in the sex chromosome and in the autosomes of *Drosophila* follows a typical Van't Hoff curve. Taking into account the duration of development, it has a temperature coefficient in the neighborhood

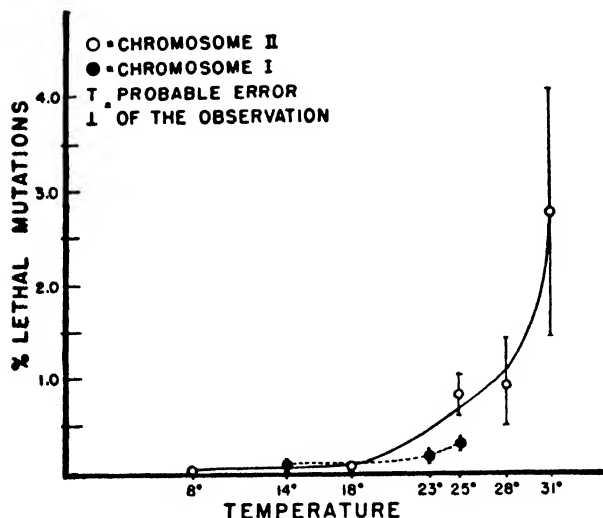


FIG. 2. Mutation and temperature.

of 5, which suggests that mutations of this sort are of the nature of biochemical reactions.

THE REVERSIBILITY OF LETHAL MUTATIONS

When a lethal is indicated in one of our tests we always check it by making two cultures from the heterozygous sibs (cf. fig. 1). Its presence is then confirmed by finding a second generation in duplicate showing at least 100 heterozygous offspring, and it is then continued in culture until salivary smears are made and it is located in either the right or left arm of the chromosomes. Occasionally lethal stocks will give one or two homozygous flies per 100, usually showing some visible mutation, but such flies are invariably sterile. Strains which give more than two flies per hundred are classed as semi-lethals rather than lethals. Most of the labor of cross testing these lethals for identity has been borne by Ives, and he finds that new lethals as they appear are seldom identical with others which have appeared in the same line.

The first lethals which appeared in the temperature test series in 1938 were checked and then carried in heterozygous cultures through the summer. In the fall they were turned over to one of my students, J. B. Stearns, to be located. He found that in six of them, or 46 percent, wild type flies were appearing in something like the non-lethal 1:2 ratio. While this could be explained by contamination, that seemed unlikely if for no other reason than that it

occurred in so large a percentage, and it appeared more probable that reverse mutations of the lethal genes to normal had occurred in the heterozygous flies. This was made more probable by a series of tests of 10 wild type chromosomes from each of the reverted strains. In one such series two out of 10 gave lethal tests, and crosses of these two showed that the two were identical. This is exactly what would be expected if reversion had taken place, for the original lethal gene should still be present in steadily decreasing proportion to its new normal allele. We have found fewer cases of this sort since, but Suche, Parker, Bishop and Griffin (1939) have already reported cases of reversion of lethal genes to normal. Such reversions make it probable that a considerable proportion of the lethals are gene mutations rather than chromosome breaks or major deficiencies.

MUTATION FREQUENCY AND TEMPERATURE SHOCKS

The relation of spontaneous mutability and temperature shocks falls clearly in another category from that of constant temperature within the normal range of the species. Our own data from lethal mutations in selected Florida stock for chromosome 2 are in general agreement with the extensive series of Buchmann and Timofeef-Ressovsky (1936) on chromosome 1. But here it must be the shock and not the expression of the Van't Hoff rule which determines the increase in mutation frequency, since as already noted, a short exposure to low temperature brings about a change in the same direction as increased temperature. The most extensive evidence for the effect of low temperature shock is that of Birikina (1938), who tested both the first and second chromosomes. A comparison of these data is given in Table 3.

Previous cited data have shown that continuous low temperature reduces the number of mutations even though it greatly lengthens the period of development, but Birikina's data indicate that a brief transition from 23° to below freezing gives a marked increase. So also the brief exposure to high temperature would hardly be expected to cause any observable increase if it were the temperature alone which was responsible. The relation between temperature shocks and mutation frequency cannot be said to have a temperature coefficient. The relation disclosed corresponds very closely with the effect of temperature on crossing over discovered by myself many years ago (1917), when it was found that both heat and cold produce an increase. As with crossing over, mutations caused by temperature shocks may be produced by temporary changes in physical state in which the most easily imagined result would be chromosome breakage.

TRANSLOCATIONS AND TEMPERATURE

This line of reasoning suggested that it might be possible to distinguish between the effect of tem-

TABLE 3. TEMPERATURE SHOCKS AND LETHAL MUTATIONS.

Treatment	Chromosome 1 (Initial indicates investigator—figures as in Table 2)				Chromosome 2			
36°-38° 12-24 hours 3 day larvae	B. & T.	11,687	34	<div>0.29 ± .03</div>	P	452	6	<div>1.33 ± .35</div>
Controls 22°-25°	B. & T.	6,495	10	<div>0.15 ± .048</div>	P	979	8	<div>0.82 ± .19</div>
	Birkina	9,695	21	<div>0.22 ± .03</div>	Birk.	3225	27	<div>0.83 ± .11</div>
-6° 25-40 min. larvae	Birkina	9,430	66	<div>0.69 ± .05</div>	Birk.	2523	62	<div>2.45 ± .21</div>

perature as such, and temperature shocks, by testing for obvious breaks, such as translocations. Some indication that translocations might be involved in the lethal mutations following shock had been furnished by a greater than expected number of simultaneous chromosome 2 and 3 lethals in our first series of tests. I have tested this possibility during the past few months with the assistance of one of my students, Ehrenfeld. With a mating scheme similar to that shown in Figure 1, we have isolated male flies each with one heated or one shocked second and third chromosome and then tested for free assortment or association between the two chromosomes. Obviously, the method picks out only this one of several possible translocations. The method is similar to that used by Berg (1938) in her studies of translocations produced by radiation. Stern (1934) tested for spontaneous translocations among the offspring of 900 triploid females and found none. Our results are also largely negative as shown in Table 4.

Ehrenfeld's tests were made using translocation free isogenic ++ second and third chromosomes of the selected Florida stock, while mine were similar except that the second chromosome carried the dumpy and black genes. The test temperatures were applied to the larvae in successive broods of the first generation and the counts made in the third. As the table shows, one translocation only was found in the control series. This was checked for a second generation and showed low viability, but was lost through an accident before salivary smears could be made. The tests were, however, sufficient to establish that the maximum frequency of spontaneous translocations is less than that of sex-linked lethals. Apparently the production of translocations is not appreciably influenced by temperature, nor is it increased by temperature shocks. We are, therefore, justified in concluding that the increase in lethal

mutation frequency following shock cannot be caused by major two point breaks which result in translocations.

TABLE 4. SPONTANEOUS TRANSLOCATION FREQUENCY
Cy/+, *D/+* ♂ × *Cy/Pm*, *D/Dfd* ♀

	14°	25°	28°	Shock 12–20 hrs. 36.5° on 3 day larvae
Ehrenfeld series (+/+)				
No. tests—	0–0	87–0	97–0	131–0
No. transloc.				
Plough series (<i>dp b/+</i>)				
No. tests	92–0	91–1	101–0	77–0
No. transloc.				
Totals	92–0	178–1	198–0	208–0
Grand total	676–1			
Max. % transloc.	0.15			

GENETICALLY DETERMINED DIFFERENCES IN MUTABILITY

While our data indicating a relation between temperature and mutation frequency have been self-consistent as long as the tests were made on the same unselected stocks, we immediately began to get unexpected irregularities and even contradictions as soon as tests with other stocks became available for comparison. Other investigators have found the same inconsistencies and these have sometimes led to doubt that temperature or temperature shocks had any effect on mutation frequency at all. For instance, as already cited (Table 2) Timofeeff-Ressovsky and Buchmann found a lethal mutation frequency for chromosome 1 of 0.15 percent at 22° and of 0.33 percent at 28°, yet our 25° frequency (Table 1) was 1.09 percent. And for chromosome 2

we found at 28° a frequency of 0.69 in one stock and of 1.74 percent in another, a difference greater than that produced on the first by high temperature. These results all pointed to genetic differences in mutability between different stocks, and we began a series of tests of different stocks at constant temperature (25°). At about the same time Demerec (1937) recognized the need of similar comparisons and published an extensive series of Chromosome 1 tests on various laboratory stocks. Table 5 brings together a selected set of values from lethal-free parents taken from our own data and other published results for both chromosomes 1 and 2.

TABLE 5. SPONTANEOUS MUTABILITY IN DIFFERENT UNSELECTED STOCKS AT 22°-25°

Investigator and stock	Chromosome 1			Chromosome 2		
	(Figures as in Table 2)					
MULLER 1928	3935	4	0.03	6286	12	0.19
BUCHMANN & TIMO- FEEFF-RESSOVSKY 19:6	6495	10	0.15			
DEMEREK 1937						
Florida	2108	23	1.09			
Wooster O	1266	8	0.63			
Formosa	2054	8	0.39			
Swedish B	1627	3	0.18			
Oregon R	3049	2	0.07			
PLOUGH, CHILD and IVES						
Florida #10	916	10	1.09	516	9	1.74
Lausanne	955	2	0.21	436	3	0.69
Belfast, Me.				189	2	1.06
So. Amherst				745	13	1.74
BIRKINA 1938	9695	21	0.22	3225	27	0.83
ZUITIN 1940 (lethals only)						
Leningrad "lab line"	8614	14	0.16			
Sukhumi "laboratory"	2309	24	1.04			
Ordjonikidze "lab"	2348	8	0.35			

Muller's data were collected using methods which may not be quite comparable, therefore they need not be considered in the present discussion. Comparison of the lethal frequencies shows that different stocks vary from 0.07 percent (Oregon R) to 1.09 percent (Florida) for chromosome 1, and between 0.69 and 1.74 percent for chromosome 2. The differences are not all statistically significant, but that similar values are secured by different tests of the same stock under similar conditions is indicated by the exact agreement between Demerec's and our values for the Florida stock (1.09). Such a comparison makes it quite clear that stocks do differ significantly in spontaneous mutability, and that estimates of the effect on mutation rate of external agents whose effect may be of a low order of magnitude must be made at the same time and on the same stock. In the few cases where the same stock has been tested in both chromosomes it is indicated that high muta-

bility in one chromosome is correlated with high mutability in the other.¹

THE EFFECT OF SELECTION ON THE MUTATION RATE

If the differences in mutation frequencies between different stocks are genetically determined, it would seem that they should be influenced by selection. Thus it should be possible to select out a low mutating line from a high mutating stock. Indeed exactly this explanation has been given by Berg (1938) for the relatively much higher mutability shown by chromosome 2 in contrast to chromosome 1. Comparisons of the stocks whose mutation frequencies in both chromosomes are given in Table 5 has already indicated that the Florida stock gave an abnormally high mutation frequency for chromosome 1. Yet this was the comparison used in Table 1 to show that the mutation frequency was in proportion to the relative numbers of genes (1-1.6). Actually both the Lausanne stock and the stock used by Birkina gave much higher relative frequencies for chromosome 2 than the comparative gene numbers of 1-1.7 would lead one to expect. (Lausanne 1-3.3, Birkina's stock 1-3.8.) Berg found a similar difference in lethal mutation frequency following X-radiation. She suggests that this is due to the selecting out of lethal producing X chromosomes in males in every generation, while lethal-producing second chromosomes are subjected to much less stringent selection.

In our studies of the Florida stock the data suggest that stringent selecting out of lethal or low producing lines does result in a marked lowering of the mutation rate per generation. In the earlier stages of our work we had supposed that we could secure the most accurate index of mutation rate per generation with the least labor by isolating lethal-free chromosomes according to the method indicated in the diagram (column 4, fig. 1), and then continuing the same lines, testing generation after generation. Our practice was to test 100 chromosomes, continue the 10 lines with the most offspring by heterozygous sister matings, eliminate the lines which showed lethals, test and continue. The results of this selection experiment are tabulated in Table 6. Generations number 1, 10 and 27 showed the lethals which had accumulated in the interval of untested mass cultures. The mutation frequency indicated by the sum of tests in generation 11 through 14 is 1.64 percent, while that for generation 28 through 33 is 0.78 percent. Thus four generations of selection brought this series of high mutating lines

¹ In addition to the data cited in Table 5, Olenov et al. (1939) list tests which are stated to be from lethal free second chromosome in three different stocks. Two of these gave such a high percentage (5.9 and 4.6) that they can hardly be accepted without confirmation.

TABLE 6. FREQUENCY OF CHROMOSOME 2 LETHALS IN SUCCESSIVE GENERATIONS AFTER ELIMINATION OF LETHAL PRODUCING CHROMOSOMES

	Generation tested	No. chrom. tested	No. lethals	% lethals	Totals
Oct. 1936	1 accumul.	92	5	5.43	5.43 ± 1.56
8 generations in mass cultures					
Mar. 1937	10 accumul.	51		3.9	3.92 ± 1.82
Apr. 1937	11	68	0	0.00	1.64 ± .49
	12	106	3	2.83	
May 1937	13	65	2	3.08	
	14	66 (305)	0 (5)	0.00	
12 generations in mass cultures					
Dec. 1937	27 accumul.	80	5	6.25	6.25 ± 1.82
Jan. 1938	28	127	1	0.78	0.78 ± .23
	29	141	1	0.71	
Feb. 1939	30	135	1	0.73	
	31	103	1	0.97	
Mar. 1939	32	45	1	2.22	
	33	92 (643)	0 (5)	0.00	

down to about the level shown by most of the other stocks listed in Table 5.

Whether one or several genes are involved in mutation frequency cannot be determined from these experiments, but they do indicate that both genetic and environmental factors are concerned in the general mutation rate throughout a whole chromosome.

OTHER FACTORS INFLUENCING SPONTANEOUS MUTABILITY

A considerable number of other agents both external and internal have been cited in the literature with apparently statistically ample data to indicate an effect on spontaneous mutability in *Drosophila*. Among these are chemical substances in the food as iodine (Sacharov 1936), copper sulphate (Magrzhikovskaja 1938), ammonia (Lobashov 1937). Zuitin in a series of papers (1938, 1940) gives evidence of increase in mutation frequency resulting from repeated minor changes in temperature, such as occur in moving cultures from an outside orchard into the laboratory. All these agents may eventually be accepted as influencing mutability, but they must first be confirmed by other workers under stringent conditions of test. It is proper to point out that large numbers do not without confirmation constitute proof of an effect on mutability, especially since they require the combined labors of a number of assistants, who may not be equally skilled in methods or interpretation. The effect of selection demonstrated in Table 6 shows the possibility that the mating scheme may give a false appearance of positive or

negative environmental effects on mutation. At the risk of having the argument turned on my own data I should like to quote a sentence from Auerbach's recent (1941) report (p. 263):

"Reliable figures for spontaneous mutation rate can only be expected by using devices for maintaining such uniformity, by randomizing the remaining variations through the use of a fairly large number of parents, by taking precautions against sources of error through non-disjunction, crossing over and the like, and, above all, by working with sufficiently large numbers."

Auerbach's own work offers one of the most interesting suggestions of an internal (physiological?) influence on mutation frequency in her clear demonstration of a marked excess in the number of spontaneous sex-linked lethals occurring in male germ cells as compared with female germ cells. It should be pointed out, however, that in the two experiments where the differences are most striking, the mutation frequency is unusually high. In checking over our second chromosome data, I find that in the first series of tests on the Florida stock (Table 6, generations 10-14) we used heterozygous males and females indiscriminately, but our records do not allow us to determine which lethals came from male and which from female original heated parents. In any case this series gave the highest rate of spontaneous chromosome 2 lethals so far found. In all of our later cultures we have used only heterozygous males, since it is easier to get the required virgin females from the control rather than the experi-

mental line, so our data are inconclusive on this point. The relation should certainly be investigated in chromosome 2.

In the tests which Ives and I made of the total mutation frequency, we did compare the numbers of mutations found following temperature shock applied to the male and female parents separately. Our record shows (Table 9, Plough and Ives 1935) that mutations were about five times more frequent than the controls when either the male or the female parent alone was heated, and about nine times greater when heated chromosomes came from both parents. Since this series involved tests of over 100,000 flies, it appears that the same increase in mutation frequency follows shock treatment of either the male or the female germ cells. These observations certainly throw the weight of evidence against the acceptance of Auerbach's data indicating that more spontaneous lethals occur in male than in female cells.

SPONTANEOUS MUTATION AND CHANGES IN WILD POPULATIONS

The facts which have accumulated concerning spontaneous mutability have certain implications of theoretical interest. First, agencies which increase mutability are of some importance in natural evolution. Wright's extensive mathematical analyses of the automatic processes involved in the shifting gene equilibrium within a population have already received some experimental verification on *Drosophila* populations by the work of Dubinin, of Dobzhansky, and of Ives. Since the data indicate that, within the limits of the temperature tolerance of the species, the higher the temperature the greater the number of mutations, it follows that the whole interacting system of forces involved in fluctuating gene frequencies will be speeded up when the temperature is elevated. Thus other things being equal any factor which increases mutation frequency, up to a certain point at least, may be expected to speed up the evolutionary process without influencing its direction.

Zuitin in a series of papers (1938, 1940) has given evidence that a succession of minor temperature changes such as occur in any natural environment cause slight but significant increases in frequency of chromosome 1 lethals, and he revives an earlier suggestion of ours, that temperature changes may be considered as the chief agent in maintaining the basic rate of mutation, or genetic variance, in nature. It is not certain from Zuitin's data that his differences may not equally well be explained by genetic differences in the stocks used. But in any case the demonstration that mutations occur even under the most constant conditions, and that spontaneous mutability has a temperature coefficient of its own, appears to eliminate temperature shocks, or any other external agent as the major causal agent in basic mutability. No stock of *Drosophila* can be genetically static in nature no matter how constant its environment may be. The automatic genetic

processes of inbreeding and selection may produce a gene equilibrium, but it will be a dynamic equilibrium and not a static condition due to the cessation of the mutation process.

SPONTANEOUS MUTATIONS AND THE GENE-CHROMOSOME PROBLEM

It would be presumptuous to suggest that a study of spontaneous mutability can offer crucial evidence on the nature of the gene, or the problem of distinguishing gene from inter-genic chromosome material. Nevertheless, the data reviewed here do have some bearing on these problems, even though the radiation work in conjunction with ultraviolet absorption studies appears more likely to give the final answers.

The increased mutation frequencies caused by development at high temperatures and that produced by temperature shocks are of about the same order of magnitude, but it is clear that they must come about in different ways. The temperature is not the essential factor in the shock treatment because its duration is too short, and because low temperature shock appears to be as effective as high. This suggests that the temperature intensity relation expresses itself in the chemical process of gene mutation, while the shock produces an effect on some other elements in the system with which a temperature coefficient is not involved. This latter may well be the inter-genic substance.

In order to prove that these agents act on different elements in the gene-chromosome system it would be necessary to show that the lethals which appeared following the different treatments differ in kind. This we have not succeeded in doing. Tests of lethals appearing in our laboratory show that they occur with equal frequency in the two arms of the second chromosome. Hence they are probably scattered through the chromosome at random as the lethals analysed by others have proved to be. While some of our lethals have been found to be associated with inversions, it is quite possible that these were present before the lethal mutations occurred. Sakharov and Naumenko (1936) found more chromosome aberrations among induced X lethals than among spontaneous ones, and this is the sort of result our data would lead us to expect, but Slizynski (1938) found the contrary result with about the same percentage of visible deficiencies in each. In addition, our translocation tests (Table 4) showed the major two point aberrations to be almost non-existent as spontaneous mutations under any of the conditions tested.

In spite of this negative evidence there still exists reason to believe that it is possible to discriminate between spontaneously occurring gene mutations, which are increased in frequency by constant high temperature, and minor aberrations or deficiencies induced by temperature shocks. This is to be found in a tabulation of the shock effects showing genetically different mutation rates. These are given in

Table 7. The newly isolated South Amherst stock gave no increase following shock treatment, while the selected Florida showed an increase. In general, stocks with high mutation rates give no response to shock, those with low rates do respond. The point is even more apparent if we compare the total of unselected stocks with that from selected stocks from which the genetic factors for high mutability have been removed. Unselected high frequency stocks gave no increase in mutation frequency following shock treatment, while selected stocks with a low frequency showed a two-fold increase. Incidentally, it may be pointed out that totalling all these tests would make it appear that shock treatment has no effect whatever, in spite of the clear contrary evidence. By gathering enough data from either line it would be possible to prove either that temperature shocks do or that they do not cause an increase in mutability; and just such conflicting reports are found in the literature. The conclusion suggested by the data in Table 7 is that genetically determined high mutability and the increase due to shock can-

Since the increase in mutation rate due to constant temperature appears largely independent of the stock, we appear to have some basis for the belief that we can roughly distinguish between two sorts of spontaneous mutations: 1) those influenced by temperature, and 2) those influenced by shocks and by the genetic factors. These may provisionally be identified as 1) gene mutations, and 2) breaks or injuries to the inter-genic substance of the chromosome. We have at present no better reason for so identifying these two classes than the temperature relation in the one case, and the lack of it in the other.

Chromosome breakage is known to be increased by low temperatures from the early studies of crossing over, and the recent important observations of Sax (1940) on the chromosomes of *Tradescantia* show that X-ray induced breaks are greatly increased at low temperatures if the cells are radiated at resting stage or at mid prophase, but by high temperatures at early prophase. Sax suggests in explanation "possibly viscosity changes or changes

TABLE 7. EFFECT OF TEMPERATURE SHOCKS ON MUTABILITY IN DIFFERENT STOCKS

Stock tested	Control 25°			Shock at 36.5° 12-24 hrs. on 3rd day			Diff.
	No. tested chrom.	No. lethals	% lethals	No. tested chrom.	No. lethals	% lethals	
<i>Mass culture—lethals accumulated</i>							
Fla-10	92	5	5.40	287	32	11.20	+ 5.80
<i>Lethal free parents</i>							
Fla-10	516	9	1.74 ± .38				
Fla-10 (sel. chrom.)	979	8	0.82 ± .19	452	6	1.33 ± .35	+0.51
Lausanne (Child) (sel.)	436	3	0.6 ± .26	* 87	2	2.29 ± 1.06	+1.06
Belfast, Me.	189	2	1.06 ± .50	348	6	1.72 ± 4.6	+0.66
So. Amherst, Mass. (Ives)	745	13	1.74 ± .33	500	5	1.00 ± .30	-0.74
<i>Total—unsel. chromosomes</i>	1450	24	1.65 ± .22	848	11	1.29 ± .26	-0.36
<i>Total—sel. chromosomes</i>	1415	11	0.78 ± .15	539	8	1.48 ± .45	+0.70
<i>Total all tests</i>	2865	35	1.22 ± .14	1387	19	1.37 ± .41	+0.15

not be combined. An exactly similar result was secured by Demerec with his mutable genes in *D. virilis*. It seems reasonable to suppose that both the genetic factors producing high mutability and the effects of temperature shock change the physical character of the chromosome so that it is somewhat more liable to mutation.

in the stress of chromosome coiling." That the chemical effects on mutation frequency may be of this nature is suggested by several recent studies, especially by Dotterweich (1941) who has found that flies fed on food containing jaborandi tincture (active ingredient the alkaloid pilokarpin) gave greatly increased mutation frequency following

radiation, as compared with radiated normal stocks.

Altogether the interpretation of spontaneous mutability seems to require the well authenticated gene and chromosome framework upon which modern genetics has been built. Our studies indicate that we are dealing with two separate phenomena, 1) gene mutations which are subject to the temperature intensity relation of chemical reactions, and 2) chromosome breaks—minor or major—which are not temperature sensitive, but are influenced by agents which may change the physical texture of the chromosome.

SUMMARY

The data presented in this review have brought out the following facts: 1) The frequency of spontaneous mutations in the three large chromosomes of *Drosophila* is in direct proportion to the numbers of genes in each. 2) Lethal mutation rate in chromosomes 1 and 2 is in direct proportion to temperature, and has a temperature coefficient of about five. 3) Lethal mutations may revert back to normal. 4) Temperature shocks both above and below the normal optimum produce an increase in mutation frequency in stocks which show low mutability and in proportion to the severity of the shock. Stocks showing high mutability appear to be unaffected. 5) There are clear cut genetically determined differences in lethal mutation rate between different inbred stocks. All the chromosomes in the fly appear to be affected by the genes determining high or low mutability. 6) Spontaneous translocations between chromosomes 2 and 3 are extremely rare and their frequency is unaffected by temperature, by temperature shocks, or by genetic differences in mutability. 7) Data of several investigators suggest that lethal mutation frequency may be increased by other external agents than temperature. 8) The evidence is still conflicting as to whether a sex difference exists in spontaneous mutation rate.

These various observations seem to justify certain general interpretations. 9) The chemical substrate of heredity is so set up in nature that a minimum rate of mutation will always occur. Thus increases in mutation frequency, however caused, may be expected only to speed up the automatic processes of natural evolution, not to change their direction. 10) Analysis of the agents which influence spontaneous mutability requires the classical gene-chromosome framework involving genes as entities separable from the inter-genic substance.

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DISCUSSION

IVES: I would like to point out: 1) While the data on lethals are not strongly confirmatory of the 1935 data on visibles, in the latter we were looking for lethals and not visibles. The temperature effect on lethals and visibles may differ. 2) In comparing our 1935 data with Auerbach's it should be borne in mind that her sex difference in the lethal rate was concerned with sex-linked lethals, while the Amherst data are for the most part on sex-linked and autosomal visibles. The situation may be different for these two types of mutants. 3) It is not clear how selection in four generations could reduce the mutation rate by one half. This is not simply removal of a few mutating loci; for assuming as few as 100 loci to be contributing to the lethal frequency, only about six of these would be eliminated in as few as four generations. Obviously, this would not have reduced the observed rate of lethal mutation detectably.

DEMEREC: What evidence does the Amherst

group have on Goldschmidt's theory that mutation is due to chromosomal rearrangement and not gene changes, and that high mutability lines carry aberrations?

PLOUGH: Goldschmidt interprets one of our cases this way, but I do not agree. We cannot check this case because its mutability has since reduced. In a very few cases we have found an inversion present in one arm of a lethal second chromosome. It may have been there when the experiment began but some of the mutants were quite independent. I think Goldschmidt is stretching the data to meet his ideas adopted *a priori*.

DEMEREK: Goldschmidt says that if mutants obtained from our high mutability stock were analyzed cytologically, they would prove to contain aberrations. Actually some of this analysis was done by Slizynski, who studied salivary gland chromosomes of induced and spontaneous lethals. The spontaneous lethals were all from the high mutability stock, and no aberrations except small deficiencies were found.

PLOUGH: There are no obvious chromosomal rearrangements in my lethals reported in this paper, but I am not sure about the finer salivary gland chromosome details.

IVES: We tested them for crossing over with 2ple, and found no evidence of small rearrangements, but only an occasional large inversion, probably already present when the lethals occurred.

MULLER: The frequency of spontaneous origin of translocations has been investigated by Altenburg (1928), who tested over 4000 chromosome combinations from ultraviolet treatment without finding any translocations. These may be considered as controls, since ultraviolet can hardly be assumed to suppress translocations.

PLOUGH: Stern (1934) made 900 tests on triploids and found no spontaneous translocations. It

is true that inversions may be present and this is now being tested.

IVES: Translocations do occur without specific agents, for example, one which I happened to observe because of its position effect. It appeared in the 25° C control series of my experiments with scute (1939).

FANO: It has been shown by Timoféeff that there is an increase in the frequency of mutation in sperm depending on age. The interpretation depends on the time within the life cycle which is considered to be available for such an accumulation. Are selections occurring during the developmental period?

MULLER: Auerbach and I had planned to test these questions when the war began and made such large-scale work impracticable.

FANO: If mutations occur early in development, there should be a number of identical mutations in the progeny.

CHILD: Lethals do occur early since identical lethals have been found in a number of sperm in the heat treated series from the same parent. Such a group was recorded as one lethal and not several.

SCHULTZ: Patterson irradiated males and transferred them daily to new females; he found the first brood most severely affected. The oldest sperm seem therefore the most sensitive. Are there further data on this?

CHILD: This also happens following heat treatment. At first the males are sterile, but after a few days, functional sperm are obtained. Of course this may mean that the sperm are killed, and that earlier stages have now metamorphosed.

DEMEREK: The data of Kaufmann and myself in *THE AMERICAN NATURALIST* (1941) indicate that X-rays produce a similar effect on all sperm which were mature during the treatment and that it takes a male about 12 days to exhaust the supply of mature sperm.

THE GENETIC CONTROL OF MUTABILITY IN MAIZE

M. M. RHOADES

Genes with a high spontaneous mutation rate are called "unstable" or mutable genes. Some investigators, notably Correns and Goldschmidt, hold that these highly mutable genes are sick or diseased—in other words their high mutability arises from some intrinsic and presumably fundamental difference not shared with more stable loci. Goldschmidt believes that any conclusions reached concerning the phenomenon of mutation from a study of unstable loci are not applicable to an understanding of the mutation process in general but apply only to the unstable loci. Inasmuch as mutable loci offer one of the most favorable objects for the study of mutation it is essential that this supposed difference between stable and unstable genes be subjected to careful scrutiny. The purpose of this paper is to present previously published data bearing on this question as well as some unpublished data recently obtained.

The spontaneous mutation rate of various genes in maize differs widely. On the one extreme there are those loci with certain alleles having a very low mutation rate, measured in terms of the rate of change of the dominant allele to a recessive condition. Stadler measured the mutation rate of seven genes in maize, which were unselected save that they affected the color or composition of the endosperm and aleurone. Four of the seven tested genes, namely *Wx*, *Sh*, *Y* and *Su*, proved to be extremely stable and may be considered examples of genes with low spontaneous rates. The *R* allele had a mutation rate several hundred times greater than that of the four genes listed above. It mutated to the *r* allele at the rate of 492 per million gametes while no mutations of *Wx* to *wx* occurred in 1,503,744 gametes and only 3 of *Sh* to *sh* were found in 2,469,285 gametes. The mutation rates of *Pr* and *I* were intermediate between these two extremes. Stadler's data indicate that there is no sharp demarcation between genes in terms of mutation rate although too few were studied to establish this definitely.

In addition to the foregoing data on the frequency of germinal mutations there exist in maize a number of cases of variegation in which it has been demonstrated that the mosaic tissue is due to the instability of certain genes. The classic example of an allele with a high spontaneous mutation rate is the unstable *P^{vv}* allele which mutates with a high frequency to a dominant allele producing color in the pericarp. Emerson (1914, 1917, 1929) presented conclusive evidence that the color change from colorless to colored pericarp found in the variegated strains of maize arose by mutation. Since variegation is a widespread phenomenon throughout the plant kingdom Emerson's demonstration that variegation

in maize pericarp color is due to the mutation of the *P^{vv}* allele to *P* suggests that mutation may be responsible for many cases of variegation. Sturtevant and Beadle (1939) are of the opinion that Emerson's work with variegated pericarp constitutes one of the mile posts in the progress of genetics—an accolade to which the writer wholly subscribes, since Emerson was the first to prove that variegation is the result of a genetic change which is heritable when it occurs in germinal tissue. Demerec (unpublished) has analyzed a situation in maize in which a waxy strain has many sectors showing the dominant starchy condition and has shown that the waxy-starchy mosaicism is due to the mutation of an unstable *wx* to a stable *Wx* allele. Hadjinov (1939) has described an allele at the *R* locus which apparently mutates from a dominant to a recessive condition with high frequency. The published data on this case are not completely convincing, however.

Although the *P^{vv}* and mutable *wx* genes are considered as unstable recessives which frequently mutate to dominant alleles there exist at both these loci other recessive alleles which have never mutated. One should speak of stable or unstable alleles rather than of loci since at certain loci both stable and unstable alleles occur.

There exists then in maize a wide, and possibly gradual, difference in the frequency of mutation of various genes. The writer agrees with Demerec's statement that there is no discontinuous range in mutation rate frequency and that the mutation for different genes may be considered as falling at various places along a continuous spectrum. If this be true it follows that mutation of an unstable gene is not a phenomenon *sui generis*. The writer has recently (1936, 1938, 1939) described a situation in maize where an extremely stable gene is made highly unstable when subjected to a specific genetic environment. The results previously published are briefly presented below.

The *A* locus in chromosome 3 is concerned with anthocyanin formation in the aleurone, plant and pericarp colors. Of the four alleles at this locus described by Emerson and Anderson (1932) the lowest member of the allelic series is the recessive *a* allele which when homozygous produces colorless aleurone, brown plant color, and a recessive brown pericarp. Stocks homozygous for *a* and the complementary dominant factors necessary for aleurone color, *A2*, *C* and *R*, are known as *a*-testers and have been extensively used by maize geneticists for many years. In so far as the writer is aware the *a* allele has never mutated to a higher member of the allelic series; there is every reason to believe it

has an extremely low mutation rate. These *a* strains so widely used in genetical experiments, and indeed all strains of maize so far tested save one, are homozygous for the recessive allele *dt* which is in chromosome 9. However in a stock of Black Mexican sweet corn the dominant allele *Dt* was discovered. The same *a* alleles so stable when present in the same nucleus with *dt* become highly unstable when the *Dt* allele is substituted, and mutate to various

indicating that mutations are most likely to occur at a definitely restricted stage in the development of the aleurone. No extremely small mutant areas are found in the aleurone and large ones are rare.

The phenotypic effect produced by the mutations of *a* in the various tissues is given below. The outline of the colored areas indicates the planes of cell division followed by the descendants of the mutated cell.

	Aleurone color (with <i>A2</i> , <i>C</i> , <i>R</i>)	Plant color (with <i>B</i> , <i>Pl</i>)	Pericarp color (with <i>P</i>)
<i>A dt</i> or <i>A Dt</i>	colored	dominant purple	dominant red
<i>a dt</i>	colorless	recessive brown	recessive brown
<i>a Dt</i>	colorless background with numerous small, round colored areas	brown background with narrow, longitudinal purple stripes	brown background with red stripes converging at the point of silk attachment

members of the allelic series. Mutations of *a* in the presence of *Dt* occur in every tissue in which this locus is concerned with anthocyanin formation, but the size of the mutant areas is small, indicating that the mutations occur late in development in all tissues. Especially in the aleurone is this true, and the slight variation in size of the colored areas further suggests that the time of mutation is restricted to a fairly definite stage in the maturation of the tissue. It is as if the cellular environment conducive to mutability becomes effective at a relatively late stage for each tissue. Since the tissues in which mutations of *a* can be detected mature at different times during the development of the plant, and since in each tissue mutations occur late in ontogeny, it is probable that mutation takes place when each tissue has reached a certain physiological condition as it approaches maturity. Occasionally a mutation occurs early enough so that a large portion of the aleurone is colored, but the great majority of the colored spots are of the same approximate size,

The above color changes in the aleurone, plant and pericarp colors are indicative of the mutations of the *a* allele to *A* but do not in themselves constitute proof that mutations have occurred since other hypotheses could be invoked to account for the colored areas. The direct proof of the mutation hypothesis was possible because in the germinal tissue an occasional mutation occurred which could be subjected to genetic tests in subsequent generations. The proof of the mutation hypothesis is discussed in some detail in the writer's 1938 paper.

It has been demonstrated that when a mutation of an *a* allele occurs in a cell of *a a Dt Dt* constitution there is no accompanying change or mutation of either of the *Dt* alleles. The *Dt* allele acts as a catalyst since it stimulates the mutability of *a* but remains unchanged when the mutations take place.

Since the mutability of the *a* allele is under the control of *Dt* the classification of *a* as a mutable or stable allele depends wholly upon the presence or absence of *Dt*. If all maize stocks were homo-

TABLE 1. EFFECT OF DIFFERENT DOSAGES OF *Dt* ON THE MUTABILITY OF THE *a* ALLELE

Pedigree	Mean number of mutations per seed		
	<i>Dt dt dt</i> class (1 <i>Dt</i>)	<i>Dt Dt dt</i> class (2 <i>Dt</i>)	<i>Dt Dt Dt</i> class (3 <i>Dt</i>)
6134-13 × 6131-7 reciprocally	6.8	19.5	—
6134-6 × 6131-14 "	5.9	19.6	—
6134-1 × 6131-2 "	7.8	19.9	—
6134-2 × 6131-9 "	9.1	23.9	—
6385-24 × 6386-13 "	6.7	24.9	—
6385-9 × 6386-19 "	8.3	26.6	—
6389-11 × 6390-17 "	8.4	24.1	—
7261-11 × 7260-6 "	6.1	24.0	—
7261-2 × 7260-1 "	6.0	17.2	—
	Mean ratio for 1 <i>Dt</i> : 2 <i>Dt</i> = 7.2:22.2		
6131-18 selfed	—	—	110.1
6131-8 selfed	—	—	126.7
6386-2 selfed	—	—	128.7
	1 <i>Dt</i> allele gives 7.2 mutations per seed. 2 <i>Dt</i> alleles give 22.2 mutations per seed. 3 <i>Dt</i> alleles give 121.9 mutations per seed.		

zygous for *Dt* then the *a* allele would be classed as a mutable gene. It is possible that some cases of unstable genes might prove to be stable in the absence of certain factors affecting or even controlling their mutability.

DOSAGE EFFECTS

As the aleurone is triploid tissue, two of the haploid sets of chromosomes being contributed by the embryo sac and one coming from the pollen grain, it was possible to determine the frequency of mutation when one, two and three *a* alleles were present. The test of the dosage effect of *a* was technically feasible by the use of the *a^p* allele which produces a pale colored aleurone, recessive to the deep color of the *A* allele but dominant to the colorless condition of *a*. The mutability of the *a^p* allele, or of any allele at the *A* locus save *a*, is not affected by *Dt*. Seeds carrying *Dt* and heterozygous for *a* and *a^p* have the pale color due to the *a^p* allele and in addition show deep colored dots which are produced by mutations of the *a* alleles to *A*. By substituting the *a^p* for the *a* allele it was possible to obtain seeds with one, two and three *a* alleles. Extensive data presented in the 1938 paper show that the effect of increasing the dosage of the *a* allele is a linear or arithmetic increase in the number of mutations. Seeds with one *a* allele have one-half as many mutations as do seeds with two *a* alleles, and one-third as many as seeds with three *a* alleles.

Previous data published in 1936 show that a non-linear effect is obtained with different doses of *Dt* while the dosage of *a* is held constant. However the later demonstration that several modifying factors exist which affect the *a-Dt* reaction made it necessary to repeat the experiment on the dosage effect of *Dt*, using lines which were relatively isogenic. Such a stock was obtained through repeated self-fertilization of a *Dt dt* strain; heterozygous *Dt dt* seed being used in every generation to further the inbreeding. After five years of selfing the *F₅* seed was classified into *Dt Dt*, *Dt dt* and *dt dt* classes. For the dosage relation between one and two *Dt* alleles exact reciprocal crosses were made between *Dt Dt* and *dt dt* individuals. It was necessary to self *Dt Dt* plants to obtain data on the effect of three *Dt* genes. The data obtained are presented in Table 1. At least 50 seeds were used for each determination. The figures represent the average number of mutations (dots of color) in the aleurone layer. The mutation frequency in the three *Dt* class is too low. There is considerable overlapping and fusion of colored areas with such high numbers of dots. Error also enters from the fact that an earlier mutation of one *a* allele in a cell obscures a later mutation of a second or third allele. In the case of one and two doses of *Dt* this is not a significant matter, but it should be taken into account in considering the data from three doses of *Dt*. No corrections, however, have been made to the raw data given in Table 1. Due to the extreme difficulty

in counting accurately the number of dots on seeds with three *Dt* alleles only three ears were classified. They were in no way different in appearance from numerous uncounted ears of the same constitution. The data show an exponential increase in mutation rate as the dosage of the *Dt* allele is increased. These data confirm the earlier conclusion reached in 1936 that the effect is non-linear.

MUTATIONS OF *a* TO DIFFERENT ALLELES

Emerson and Anderson (1932) described four alleles at the *A* locus. Two of them, *A* and *A^b* produce deep colored aleurone, *a^p* gives a pale aleurone color and *a* produces colorless aleurone. The effects of these four alleles on aleurone, plant and pericarp colors are shown in the upper part of the following table:

Allele	Aleurone color	Plant color	Pericarp color
Old alleles			
<i>a</i>	colorless	recessive brown	recessive brown
<i>a^p</i>	pale	red-brown	dominant brown
<i>A</i>	deep	purple	red
<i>A^b</i>	deep	purple	dominant brown
Alleles derived by mutation			
<i>A</i>	deep	purple	red
<i>A^{br}</i>	deep	purple	recessive brown
<i>A^rb</i>	deep	purple	recessive red-brown
<i>a^{br}</i>	pale	red-brown	recessive brown
<i>a^r</i>	colorless	recessive brown	recessive brown

The *a^r* allele differs from *a* in that it is more stable with *Dt*.

The *a* allele is recessive to the other alleles in all respects. The *a^p* allele is dominant to *A* in pericarp color but is recessive to it in aleurone and plant colors. The *A* and *A^b* alleles are alike in their effect on aleurone and plant color but the brown pericarp color produced by *A^b* is dominant to the red of *A*.

Germinal mutations of *a* to higher alleles can be detected in purple anthers found on *a B Pl* plants or in seeds with self-colored aleurone that are occasionally found on *a Dt* ears. All of the mutations isolated from purple anthers gave the deep self-colored aleurone similar to that produced by the *A* and *A^b* alleles, and all mutations from seeds with deep colored aleurone gave purple plants with the complementary *B* and *Pl* factors. Since the *A* and *A^b* alleles differ only in their effect on pericarp color a number of mutations identical to both *A* and *A^b* as far as aleurone and plant color are concerned were subjected to further tests. Twenty-nine such mutations have now been tested. Twenty-seven of them produced red pericarp color; they may be considered as identical to the *A* allele. One of the remaining two gave a recessive brown pericarp color with *P* in contrast to the dominant red of the *A* allele and the dominant brown of the *A^b* allele. This represents a mutation of *a* to a new, previously undescribed allele. It has been designated *A^{br}*.

The other mutation produced a reddish-brown pericarp color with *P* which is dominant to the brown of *a* but recessive to the red of *A*. This is also a new allele. It has the symbol *A^{rb}*.

The pale aleurone color produced by the *a^p* allele is clearly distinguishable from the deep color of the *A* and *A^b* alleles. Therefore it was possible to determine the frequency with which *a* mutates to an allele or alleles giving pale aleurone color as compared with the frequency to alleles giving deep aleurone by inspection of the color of the aleurone dots. In the 1938 paper data were reported showing that the frequency of mutation to alleles giving deep aleurone color was a thousand times as great as to alleles producing pale colored dots. Since these mutations occur in somatic tissue it is impossible to ascertain the nature of these mutations as far as their effect on plant and pericarp colors are concerned. One germinal mutation giving pale aleurone color was found however and tested. It was similar to *a^p* in aleurone and plant color but gave a recessive brown pericarp color instead of the dominant brown produced by *a^p*. This is also a new allele. It is designated *a^{br}*.

A number of self pollinations were made in lines homozygous for *a* and *Dt* to obtain a number of germinal mutations. Families 6641 and 6642 had a combined total of 389 ears of which 19 were segregating dotted and colorless seeds in approximately 3 : 1 ratios. Six of the 19 segregating ears were tested further and in five of them the occurrence of the colorless seeds was due to mutations of the *a* allele to a new allele or alleles indistinguishable from it save that they have much reduced mutation rates with *Dt*. In all five tested mutations the seeds homozygous for the new stable alleles appear macroscopically to be devoid of mutant colored areas. Two of these mutations have been subjected to further study. One of them appears to be completely stable with *Dt* since no mutations have been found on several hundred seeds examined under a binocular microscope. The other mutation so tested is to an allele with a markedly reduced rate of mutation with *Dt* but is not as stable as the first mutation since an average of 0.4 mutations per seed was found. The size of these infrequent dots is smaller than those on seeds with the original *a* allele indicating that the time of mutation occurs later in development. The wholly stable allele may be considered to be different from the second allele with a low but still detectable mutation rate but these two as well as the other three cases have been, pending further study, collectively designated by the symbol *a^s*.

The sixth tested ear proved to be heterozygous for *Dt dt*. It is possible that the *dt* allele came from contamination rather than from mutation of *Dt* to *dt* but no such explanation can account for the presence of the *a^s* alleles since all *a* stocks available to the writer were mutable with *Dt*.

It is highly probable that the *Dt* allele caused the

mutations of *a* to *a^s*, since the observed mutation rate is so great that the *a^s* allele would have replaced the *a* allele in the genetic stocks if this mutation occurred with *dt*, and all *a* stocks are, as previously stated, mutable with *Dt*. It is possible that the *a^s* mutations observed in families 6641 and 6642 stem from only two mutations occurring in sporogenous cells which gave rise to a considerable number of the sex cells but this is not likely since the wholly stable allele and the allele with a low mutation rate came from the same family.

The relatively high frequency of the *a^s* alleles indicates that mutations to these alleles occur more frequently than to other allelic forms. While a relatively small number of germinal mutations producing color of one grade or another have been tested it appears that mutations to the *A* allele occur with the highest frequency since 27 of the 29 tested mutations giving deep aleurone color proved to be to this allele and the frequency of mutation to alleles giving pale colored aleurone is much less than to alleles giving deep color. Since the *a^{br}*, *A^{br}*, and *A^{rb}* alleles have been found only once nothing can be said about the relative frequency with which they arise.

A total of five different alleles have been found in these experiments. Four of these alleles are new, previously undescribed ones and it may be expected that additional new alleles will be found as these studies are continued.

LINKAGE RELATIONS OF *Dt*

In the 1938 paper it was suggested that *Dt* was linked with the *C* locus in chromosome 9. This statement was based on the ratio of dotted to colorless kernels in the *F₂* of plants segregating for *A a Dt dt C c*. Since the *C* allele must be present for the expression of aleurone color, linkage between *C* and *Dt* would manifest itself as distorted ratios of dotted and colorless seeds. Linkage data of this type are unsatisfactory so that linkage tests of *Dt* with other factors in chromosome 9 were made holding the *C* allele homozygous. According to the data summarized in Emerson, Beadle and Fraser (1935) the linear order and map positions of certain genes in chromosome 9 is as follows:

<i>knob</i>	<i>yg²</i>	<i>C</i>	<i>sh</i>	<i>bp</i>	<i>wx</i>	<i>v</i>
0	2	21	24	39	54	66

The knob is located at the end of the short arm of chromosome 9 and all of the above loci except *v* fall in this arm. The linkage data of *Dt* with *Yg2*, *Sh* and *Wx* are given in table 2. These data definitely prove that *Dt* is in chromosome 9 and further indicate that *Dt* lies close to the *Yg* locus near the end of the short arm. The order is unquestionably *Dt Sh Wx* and may be *Dt Yg2 Sh Wx* with *Dt* about ten units beyond *Yg2*. Creighton found approximately two percent recombination between *Yg* and the terminal knob on the short arm of 9 so there is some doubt if *Dt* is beyond *Yg* and

if it is that the crossover distance is as great as ten units. It should be noted that in selfing a *Dt dt* plant three classes of dotted seed are obtained, that is, *Dt Dt Dt*, *Dt Dt dt* and *Dt dt dt* classes. In the latter class possessing a single *Dt* allele the mutation rate is so low that a considerable number of *Dt dt dt* seeds are classified as *dt* because no dots (mutations) are evident. This fact introduces some error into the recombination values and would tend to increase the *Dt Yg* value. Nevertheless the data indicate that *Dt* is close to the terminal knob which is presumably composed of heterochromatin. While the placing of the *Dt* locus close to the knob is probably without significance it is of interest in view of Schultz's demonstration that loci normally stable when removed from heterochromatin show a type of variegation suggestive of mutation when brought into proximity with heterochromatin. The situation here is different since the *a* allele made mutable by *Dt* is in another chromosome and both *Dt* and *dt* containing chromosomes have terminal knobs on the short arm of 9 which do not appear to differ in size.

inversions so minute as to be beyond the resolving power of the microscope we should expect to find, in some mutations at least, evidence of gross structural changes. No such aberrations have been found. Chromosomes 3 carrying *A* alleles derived by mutation are not visibly different from homologues bearing the *a* allele. Minute inversions would be more difficult than translocations to detect cytologically or genetically. Inversions, when heterozygous, should reduce the amount of crossing over between the *A* locus and neighboring genes in chromosome 3 if they are of considerable length. Unpublished data of the writer give 30 percent recombination between *A* and *lg2*. Four different mutations to the *A* allele were tested for linkage with *lg2*. All of them showed approximately 30 percent recombination, a fact which argues against the presence of any but small inversions. It is difficult to visualize any mechanism by which the *Dt* allele located in chromosome 9 could cause breaks to arise with an extremely high frequency at the *a* locus of chromosome 3 while all other chromosomes remain unaffected. And the fact that the phenomenon of

TABLE 2. LINKAGE DATA OF *Dt* WITH VARIOUS LOCI IN CHROMOSOME 9

Genes	Linkage Phase	XY	Xy	xY	xy	Total	% Recombination
<i>Dt Sh</i>	C S	679	100	156	138	1073	27
<i>Dt Sh</i>	C B	617	266	305	588	1776	32
<i>Dt Wx</i>	R S	1663	525	690	118	2996	41
<i>Dt Wx</i>	C B	682	465	472	677	2296	41

		<i>Dt Yg Wx</i> <i>dt yg wx</i> selfed			
<i>Dt Yg Wx</i>	1450	<i>dt yg Wx</i>	385	Total 2793	
<i>Dt yg wx</i>	38	<i>dt Yg Wx</i>	223	Recombination values:	
<i>Dt Yg wx</i>	360	<i>dt Yg wx</i>	63	<i>Dt Yg</i>	11 percent
<i>Dt yg Wx</i>	36	<i>dt yg wx</i>	238	<i>Dt Wx</i>	42 percent
				<i>Yg Wx</i>	37 percent

		<i>Dt Yg Sh Wx</i> <i>dt yg sh wx</i> selfed			
<i>Dt Yg Sh Wx</i>	387	<i>dt Yg Sh Wx</i>	52	Total: 779	
<i>Dt yg Sh Wx</i>	7	<i>dt yg Sh Wx</i>	84	Recombination values:	
<i>Dt Yg Sh wx</i>	59	<i>dt Yg sh Wx</i>	2	<i>Dt Yg</i>	10 percent
<i>Dt yg Sh wx</i>	2	<i>dt yg sh Wx</i>	49	<i>Dt Sh</i>	27 percent
<i>Dt Yg sh wx</i>	35	<i>dt Yg Sh wx</i>	1	<i>Dt Wx</i>	44 percent
<i>Dt yg sh wx</i>	10	<i>dt yg Sh wx</i>	3	<i>Yg Sh</i>	22 percent
<i>Dt Yg sh Wx</i>	15	<i>dt Yg sh wx</i>	9	<i>Yg Wx</i>	38 percent
<i>Dt yg sh Wx</i>	3	<i>dt yg sh wx</i>	61	<i>Sh Wx</i>	20 percent

LINKAGE RELATIONS OF ALLELES DERIVED BY MUTATION

Goldschmidt holds that gene mutations are position effects, that is that every so-called point mutation results from some chromosomal rearrangement such as translocation, inversion, etc. The writer (1938) has presented several reasons which seem to negate the possibility that mutations induced by *Dt* at the *a* locus are due to breaks in chromosome 3 at or near this locus. Unless all mutations involve

position effect is unknown in maize although many reciprocal translocations and some inversions have been studied makes it difficult to accept the view that the mutations at the *a* locus induced by *Dt* are due to minute rearrangements.

EFFECT OF *Dt* ON THE *P^{vu}* ALLELE

The *Dt* gene affects only the mutability of the *a* allele. A number of endosperm, aleurone and seedling recessives have been tested for increased muta-

bility without any indication that their mutation rates were in any way changed. The tested genes have a low mutation rate, however, and it seemed desirable to test *Dt* against a gene with a high spontaneous mutation rate such as *P^{vv}* or the unstable waxy gene. Plants heterozygous for *Dt* and possessing the variegation allele were backcrossed to *dt p* individuals. The *F*₁ seed was classified into *Dt* and *dt* classes and the resulting ears graded for degree of variegation in a similar manner to that employed by Emerson in his studies on variegation. The data obtained are as follows:

Family	<i>Dt</i> seed Number ears	Mean variegation grade	<i>dt</i> seed Number ears	Mean variegation grade
1	23	4.09	34	4.12
2	22	4.09	19	4.05
3	22	3.82	31	3.87
4	17	4.18	11	4.36
5	32	4.06	35	4.00
6	30	3.67	38	3.68
7	21	4.67	28	4.68
	167		196	
Mean grade		4.08		4.11

The above data show that there is no effect of the *Dt* gene on the mutability of the unstable *P^{vv}* allele. Similar studies were made of the effect of *Dt* on the unstable waxy allele and no change in mutability was found.

The *A2* locus in chromosome 5 closely resembles the *A* locus in its phenotypic effect. The *A2* allele gives deep colored aleurone and purple plant color as does the *A* allele and the recessive *a2* allele is like *a* in producing colorless aleurone and brown plant color. They differ in that the *a2* allele does not change the pericarp color from red to brown as does the *a* allele. Despite the similarity between these two loci the *Dt* gene does not affect the mutability of the *a2* allele.

TEMPERATURE EFFECT

Although Demerec has shown that the mutability of the unstable miniature-3 gamma gene of *Drosophila virilis* is little affected by temperature a similar experiment was conducted with the *a-Dt* material. Two different *a Dt* strains were grown at a temperature of approximately 21°C until flowering. Immediately after pollination the plants of each strain were divided at random into two lots; one lot was placed in a greenhouse maintained around 15.5°C and the second lot put in an adjoining house at or near a temperature of 27°C. The two lots of plants were left in the two houses until seed had matured. The mutation rates at the two temperature levels were determined by counting the number of dots in the aleurone of 50 seeds from each plant except for those ears marked by asterisks in the table below where less than 50 seeds were avail-

able. The data obtained from one strain are given below.

Average number mutations per seed		
	15.5°C	27°C
	50.2	2.0
	47.2	9.0
	37.5	11.5
	41.2	9.9
	44.9*	3.7
	29.5*	14.5*
		13.5
Total	250.6	65.0
Mean	41.8	9.3

The above data clearly show a depressive effect of a rise in temperature upon the mutability of *a*. However the other strain failed to show a striking effect with a rise of temperature although this statement is based on inspection of the ears as no detailed counts were made. Since the effect of an increase in temperature is beyond statistical doubt for the one strain it is possible that the second strain carried a modifying gene or genes which opposed the depressing action of temperature. This problem must be prosecuted further. Beale and Fabergé have recently (1941) described a similar depressive effect of temperature upon the mutability of an unstable gene in *Portulaca*.

DISCUSSION

It is doubtful if the mechanism by which *Dt* induces the *a* allele to become highly mutable is the same as that responsible for increased mutation rates found following treatment with short wave radiation and temperature.

These two agents cause a general rise in mutation rate while *Dt* is specific in its effect. Further, chromosomal aberrations are produced in great abundance by irradiation but none has been found in the *Dt* strains. Some investigators hold that the effect of irradiation is to cause a single alteration in the equilibrium position of an atom or electron in the gene molecule. This change in equilibrium may occur as a result of heat vibrations or of ionization following treatment with X-rays, etc. The writer is not willing to judge the validity of this hypothesis. It seems, however, that the effect of the *Dt* allele on the mutability of the *a* allele is best accounted for by assuming that *Dt* alters the cellular environment in some way so that the *a* allele becomes mutable. Simply put, the effect of *Dt* seems to be a chemical rather than a physical phenomenon. While the situation reported here probably is unique, it is possible that a similar case exists—also in maize. Emerson has shown that the *Bh* allele located in chromosome 6 causes kernels which are homozygous for the recessive *c* allele, and therefore normally colorless, to have patches of color. The *Bh* allele affects only the *c* gene and it may be that the color

found is produced by mutation of *c* to *C*. This has not yet been demonstrated.

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DISCUSSION

DEMEREC: How does the *Dt* gene affect the new alleles of *a*?

RHOADES: They are stable.

DELBRÜCK: The spots in one strain are uniform in size, in another strain they are of different sizes in the *A* material.

RHOADES: The range in all cases is small, however, though some range of size occurs in all stocks.

DEMEREC: Then modifying factors determine the time of mutation.

RHOADES: Yes, a recessive gene delays the time of mutation and the size of the spots in this strain are extremely small.

PLOUGH: In the temperature effect, are you dealing with an effect on the time of development? In other words, do spots occur at a certain time of development and has high temperature shortened that time?

RHOADES: This is possible but it probably does not account for all the difference. Fabergé and Beale have found a similar depressive effect of a rise in temperature in the case of an unstable gene in *Portulaca grandiflora* and their account will appear shortly in *NATURE*.

CHILD: You would expect this if the *Dt* gene is effective late and only during a certain period.

MULLER: The rate of development is changed by temperature but the mutation rate must be affected much more.

SCHULTZ: The temperature effect is the sort of thing obtained in the variegated types in *Drosophila*.

RHOADES: I do not think the locus of the *Dt* gene near the knob of chromosome 9 has any special significance, although this remains to be determined.

UNSTABLE GENES IN DROSOPHILA

M. DEMEREC

Twenty-seven years ago, when R. A. Emerson (1914) first suggested that instability of the gene is responsible for pericarp variegation in maize, genes were looked upon as very stable units. At that time his suggestion was considered revolutionary. Five years later a prominent geneticist (Correns, 1919) still thought that changes associated with variegation occurred too frequently to be called mutations and preferred to explain them as due to a disease of the gene. However, since that time the situation has changed and our views on the stability of a gene have been greatly modified. The evidence has accumulated indicating that changes in genes occur with an appreciably higher frequency than it had earlier been assumed; moreover it has been found that this frequency may be different in different genes, and that it may be affected by various hereditary factors.

During the past decade a great deal of experimental work has been done in connection with the problem of mutations. In the great majority of experiments X-rays and other sources of irradiation have been used. The problem of spontaneous mutability has been neglected and in theoretical considerations has not received the attention it deserves. Therefore, at this meeting where the question of the gene is being discussed from various angles, it seems appropriate to review the work dealing with unstable genes in *Drosophila*. It is my belief that these problems are intimately related to the general gene problem and that they will play an important role in the development of the gene theory.

GENERAL CHARACTERISTICS

Unstable genes have the capacity of mutating in several directions but in most cases which have been studied the mutational change goes from a recessive, usually mutant allele to a dominant, usually wild-type allele (Demerec, 1935). The changed form is, as a rule, as stable as any other wild type.

In the genus *Drosophila* four well substantiated cases of unstable genes are known, all four of them in *D. virilis*. They are, in the order of their discovery, reddish body color, miniature wings -3, magenta eye color, and miniature wings -5. In all four cases the mutants were discovered immediately after their origin from the wild type and in all four cases they revert back into the wild-type form.

Originally mutant alleles were designated by a Greek letter added to the name of the mutant, thus reddish-alpha, miniature-alpha, miniature-beta, miniature-gamma, etc. However, the use of the

Greek alphabet proved inconvenient for typing and added to the expense of printing. For these reasons it has been replaced by the Roman alphabet, as, reddish-a; miniature-a, -b, -c, etc.

Reddish (*re*) is an allele of yellow and affects both body color and the chaetae. Reddish flies have golden yellow body and chaetae while the color of wild-type flies is gray. Several alleles are known at the yellow locus, namely: *y*, *re-1a* (unstable), and *re-2* (stable).

Miniature (*mt*) flies have smaller wings than the wild type and in addition, the cell walls persist on miniature wings while on the wild-type wings they are absorbed. At the miniature locus the following alleles are known; *mt-1*; *mt-2*; *mt-3a, b* and *c*, (unstable); *mt-4*; *mt-5a, b* and *c*, (unstable); *mt-6*; and *mt-7*. Most of them can be distinguished from each other by the size of the wings.

Magenta (*m*) affects eye color, which is purplish red when compared with the red color of the wild-type eye. Two mutant alleles are known at the magenta locus, namely *m*, which is stable, and *m-a*, which is unstable.

An appreciable amount of work has been done with unstable reddish and miniature, while unstable magenta has not been extensively used because it cannot be easily classified.

PERIOD IN ONTOGENY WHEN MUTABLE GENES ARE UNSTABLE

By the size of the mutant sector it is possible to determine the time in ontogeny when a change in the gene has occurred. Thus small sectors indicate late changes while large sectors represent early changes. Material particularly suitable for such determinations are characters determining color and those affecting morphology.

Data collected in studies of various unstable genes show a great difference between them. Some genes, for example, unstable rose of *Delphinium* (Demerec, 1931), change with an approximately equal frequency throughout the development of the plant while in some other genes instability is limited to a certain period. A good example of the latter type is the unstable lavender gene of *Delphinium* (Demerec, 1931) which is unstable during very early embryonic development and during the late stage of flower development while it is stable in other stages of ontogeny.

Reddish-a of *Drosophila virilis* has the most restricted period of instability so far recorded (Demerec, 1928b). This gene is unstable only at the maturation divisions of heterozygous females, namely females which carry reddish-a in one chromosome and one of its alleles (wild-type, yellow or reddish-

1) in the other. Observations indicate that the gene is stable in all other cell divisions as well as in males and in females homozygous for reddish-a. The frequency of mutations varies from a very low rate up to 25 percent in some exceptional cases. By selecting for breeding purposes the offspring of females which gave a high mutation rate it has been possible to maintain a line which gave for 16 generations an average frequency of reversions of about three percent.

An interesting case of variation in the stability of a gene has been described in *Delphinium* (Demerec, 1931). The unstable gene for lavender flower color changes frequently into its purple allele. Two types of changes are obtained, those which cover a large sector of a plant and those which manifest themselves as very fine dots on the flowers and cover approximately one cell each. Intermediate spots do not occur. This shows that the lavender gene is unstable very early in embryonic development, probably immediately after fertilization, but that it subsequently becomes stable until the last cell division in the development of sepals and petals, when it becomes unstable again.

As mentioned above, two unstable alleles at the miniature locus are known, namely miniature-3 and miniature-5. Miniature-5 was found six years after miniature-3 in a cross with genetic markers which make it certain that the original male did not come as a contamination from miniature-3. In addition these two miniatures are phenotypically different, the miniature-5 having larger wings than miniature-3. With regard to mutability, both miniatures behave alike and thus they will not be discussed separately.

Unstable miniature alleles appear in three well defined forms or sub-alleles, *a*, *b*, and *c*, which differ from each other in the degree of mutability. The miniature-*a* form is unstable in both the germinal tissue and in somatic tissue, the *b* form is stable, and the *c* form is unstable in somatic cells but is stable in germinal cells. Miniature-*a* parents produce miniature, mosaic and wild-type offspring, miniature-*b* parents give only miniature, while miniature-*c* parents produce miniature and mosaic offspring.

Immediately after fertilization the *Drosophila* egg nucleus passes through eight simultaneous divisions, producing 256 nuclei. About five to eight nuclei then migrate to the posterior end of the egg and later form the germ-cells; the other nuclei move to the periphery of the egg and give rise to somatic tissue. Thus, the germ cells are separated from the somatic cells after eight nuclear divisions. By studying the size of mosaic spots, which represent somatic changes, and the proportion of wild-type flies among the offspring, which represent germinal changes, it has been possible to determine approximately the stage in ontogeny when the *a*, *b*, *c* forms of mutable miniature alleles are unstable. The results are shown graphically in Figure 1. These studies in-

dicate that all three forms are stable during the first eight cleavage divisions and probably during the early stages of somatic and germinal development. Only in the later stages of ontogeny does the *a*-form become unstable in somatic and germ cells, the *c*-form becomes unstable in soma only, and the *b*-form remains stable.

It is very likely that if miniature were more suitable material, other forms of unstable alleles could be detected. It is possible for example that a form may exist which would be unstable in late somatic development only and be responsible for small wild-type spots on the wings, and another form which would be unstable in early somatic de-

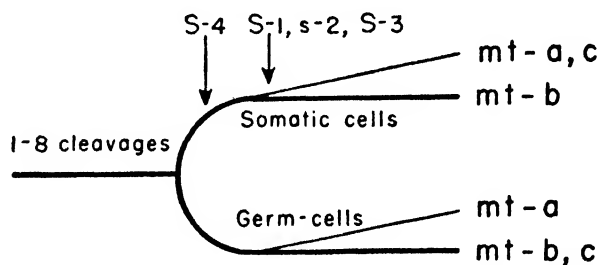


FIG. 1. Diagram indicating approximately the stages in ontogeny when *a*, *b* and *c* forms of mutable miniature alleles (*mt-3* and *mt-5*) are unstable, and the approximate period of activity of S-factors. Heavy line represents stable stage and light line unstable stage.

velopment and produce large wild-type sectors on the wings. An attempt was made to separate such forms but without success. Fine differences in spotting cannot be conveniently detected on miniature wings. It should be pointed out, however, that *a*, *b* and *c* forms are not necessarily the only forms which occur in the unstable miniature allele but they are the only forms which have been recognized so far.

EFFECT OF INTERNAL FACTORS ON MUTABILITY

Unstable genes of *Drosophila virilis* are unaffected by a number of environmental agents which have been used. Among them are a variation in temperature of 10 degrees centigrade, exposure to CO₂, and exposure to X-rays (Demerec, 1932a). However, it has been found that a number of internal factors affect the mutability of both unstable reddish and unstable miniature. For reddish it has been shown (Demerec, 1928b) that mutability is influenced by the age of the females. Among 4,993 offspring obtained from 49 females when they were less than four weeks old, 3.3 percent of reversions were found, while among 4,665 offspring obtained from the same females when they were more than four weeks old, only 1.1 percent were reversions.

Sex is another factor which affects the mutability rate. Thus reddish-*a* is unstable only in females (Demerec, 1928b), and in miniature-*c* the rate of

change in males is about twice as high as it is in females (Demerec, 1932b).

However, the most striking effect on mutability is produced by various genetic factors. For example, a specific genetic set-up, namely heterozygosity, is essential for reddish-a to be unstable. Five genes have been found which increase the mutability of unstable miniatures. None of them has any detectable morphological or physiological effect except for the increase in mutability. An autosomal dominant gene *M* affects mutability in the germinal tissues and acts in this way on miniature-a forms only (Demerec, 1930). This gene does not affect mutability in the somatic cells of either miniature-a or miniature-c forms but the increase in germinal mutability is very striking. In one experiment where the same miniature-a male mated to a female carrying the *M* gene and to another female not carrying it, among the offspring of the first mating about 80 percent were reversions while among the offspring of the second mating only 0.69 percent were reversions. *M* is not linked with *G*, *B*, *R* and *Cl* and thus it should be located in the sixth chromosome.

Four genes have been analyzed which increase the somatic mutability of miniature-a and miniature-c forms (Demerec, 1928a, 1929b, and unpublished); three of them, *S-1*, *S-3* and *S-4* are dominant and the fourth, *s-2* is a recessive. All are located in autosomes. For *S-1* it has been established that it is linked with *R* and thus located in the second chromosome; *s-2* is linked with *B*, thus located in the fifth chromosome; no linkage was observed between *S-3* and *R*, *B* or *G*, indicating that *S-3* is not located in either second, third or fifth chromosome. Linkage tests between *S-4* and *R*, *Cl* and *Gf* show that *S-4* is not located in the second, fourth or sixth chromosome.

Mosaic spots are present on about 4 percent of miniature-c flies which do not carry the mutability stimulating factors, but about 95 percent of flies show mosaics if any of these factors are present. Moreover, while usually not more than one mosaic spot is present on the flies without *S* genes, several mosaic spots are frequently to be found on the flies carrying *S* genes.

Mosaic spots on flies without the mutability factors or on flies carrying either *S-1*, *s-2* or *S-3* are, as a rule, smaller than one wing. A spot covering one whole wing is seldom to be found. However, about 3 percent of the mosaics from *S-4* cultures have one wing wild-type and the other either miniature or mosaic. This indicates that *S-4* makes miniature-a and miniature-c mutable at an earlier stage than any of the other three *S* factors. The combination of miniature-c and *S-4* genes frequently gives mosaics which have one whole wing of the wild type but it does not produce wild-type flies. This shows that these mosaics result from changes which occurred after germ cells have differentiated from the somatic cells (fig. 1).

DIRECTION OF THE CHANGES

As stated above, the most frequent change in an unstable gene goes from the mutant to the wild-type allele. In the unstable genes which were studied in *Drosophila virilis*, the frequency of changes in the material not carrying mutability stimulating factors is as follows:

reddish-a: about 3 percent

miniature-a: in germ cells about 4 percent
in somatic cells about 10 percent,
that is, about 10 percent of flies are
mosaics

miniature-b: stable

miniature-c: in soma about 4 percent

magenta-a: in germ cells about 14 percent
in soma about 0.3 percent

In all cases mentioned above the change goes in one direction only, and in no case has the wild-type allele which was derived from the mutant changed back into the mutant.

In addition to these primary changes, secondary changes from one form to another were observed in unstable miniatures (Demerec, 1929a). Thus the *a* form changes to *b* or *c*, *b* to *a* or *c*, and *c* to *a* or *b* (fig. 2). However, secondary changes are con-

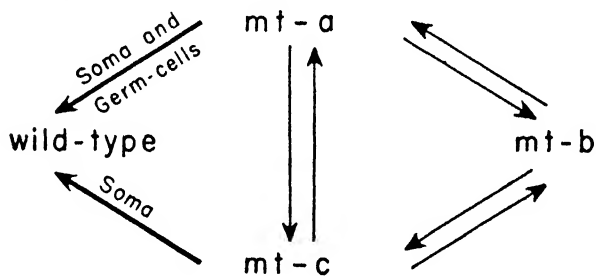


FIG. 2. Changes observed in the unstable *mt-3* and *mt-5* alleles. Primary change to wild-type (heavy lines) are irreversible and are considerably more frequent than secondary changes from one form into another.

siderably less frequent than primary ones, and they are reversible while primary changes are irreversible. In an experiment with the *a* form, one change each was observed to *b* and to *c* among 78 tests made; in an experiment with the *c* form, a change into the *a* form was observed with a frequency of about 0.3 percent; and in an experiment with the *b* form, the change to *c* occurred with a frequency of about 0.017 percent. Thus as far as the frequency of secondary changes is concerned, the *b* form is the most stable of the three. This behavior parallels that of the primary changes and suggests a connection between the two. A further connection is indicated by the fact that both primary and secondary changes are similarly affected by the mutability stimulating factors. For example, the *s-2* factor increases the frequency of changes from *c* into *a* from about 0.3 percent to about 20 percent.

It has already been mentioned that miniature-3

and miniature-5 are different phenotypically, but that both of them have an identical behavior as far as their instability is concerned. Both show primary and secondary changes. It is of interest that the phenotype is not affected in any of these changes. Miniature-3 and -5 retain their respective phenotypes even though they pass through changes to various forms.

DISCUSSION

This short review of the situation concerning unstable genes in *Drosophila virilis* and particularly concerning unstable miniature alleles shows clearly the complexity of processes connected with these changes. The fact that a gene may be stable at one stage of ontogeny and unstable at another stage as well as the other facts concerning the behavior of these genes, makes improbable the assumption that changes in unstable genes are due to mechanical assortment of sub-gene particles (Demerec, 1935). Thus the only other feasible explanation is that chemical changes are responsible for this behavior.

For the sake of argument, a gene may be visualized as a biological unit which takes up a certain region of the chromosome. It is not essential that the physical limits of a gene be well defined. It is known that adjacent genes may affect each other and it may well be that sharp limits between two genes do not exist. However, it is known that a gene retains its characteristic biological action in a variety of positions with respect to other genes, and thus it seems reasonable to look upon a gene as a biological unit.

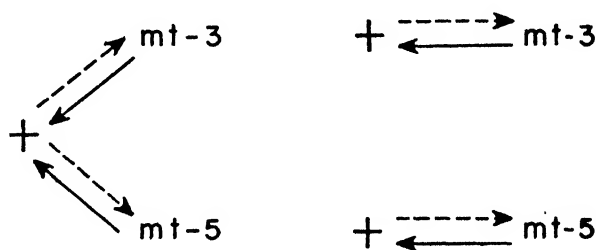
The following questions then arise: what are the chemical changes which occur in unstable genes; and is a parallel case known which would help to visualize them? Unfortunately the answer to both of these questions is negative. As yet we know very little about the fundamental chemical constitution of the gene and we know still less about chemical changes connected with the mutational processes. Therefore an attempt to explain the behavior observed in unstable genes in terms of chemical reactions would be an unprofitable speculation. I will confine myself here to a mere restatement of the observed facts and will make suggestions as to how different processes could be visualized.

Unstable genes in *Drosophila* were discovered shortly after they had originated as mutations from the wild type. They frequently revert back into the wild type. There is no difficulty in finding a parallel for such behavior since any number of reversible chemical processes are known. However, the process occurring in unstable miniature genes has two well-defined characteristics: it is reversible but the change goes from the new form to the original form only; and it is not affected by a change in temperature of 10 degrees Centigrade.

It is a relatively simple matter to explain the facts that genes are not equally stable at all stages of ontogeny and that they are readily affected by

various genetic factors. It needs only to be assumed that a certain physiological condition is necessary to stimulate the chemical change in the gene and that such a condition is met at certain stages of ontogeny only and is met with higher frequency if the individual has a certain genetic constitution.

The complexity of the situation is revealed when an attempt is made to visualize the mechanism responsible for both primary and secondary changes. It is a well known fact that several well defined alleles may be recognized at a number of loci. For example, at the miniature locus of *D. virilis* at least seven such alleles are known which differ from each other in the size of the wings. It is reasonable to assume that different chemical changes are responsible for these different alleles. It is easily conceivable that a certain chemical change in one molecular group of the gene molecule may be responsible for the *mt-3* effect while a similar or a different change in another group of the same gene molecule may be responsible for the *mt-5* effect. It is also conceivable that two different changes occurring in the same molecular group may be responsible for two different effects represented by the *mt-3* and *mt-5* phenotypes. Thus the difference between the two alleles may be due either to the qualitative differences in chemical changes or to their location on the gene molecule. In the case of *mt-3* and *mt-5*, both of which revert to wild type, this means that the original chemical changes are unstable. The situation is represented on the following diagram. The left side represents two different chemical changes in the same molecular group giving rise to two different unstable alleles; the right side represents the same end effect produced by either identical or different changes in two molecular groups. The broken arrow indicates that the change occurred only once.



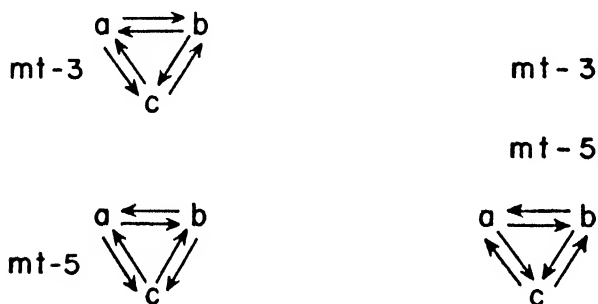
At the miniature locus, in addition to *mt-1*, *mt-2*, *mt-3*, *mt-4*, etc., the series *mt-3a*, *mt-3b*, *mt-3c*, and the series *mt-5a*, *mt-5b*, *mt-5c* are known. These *a*, *b* and *c* forms are just as distinct from each other and from *mt-1*, *mt-2*, etc. alleles as are any other two alleles, but they definitely belong to a different category than *mt-1*, *2*, *3*, etc. alleles. The *a*, *b*, *c* forms change from one into another but they retain their phenotypic status, that is, the *a*, *b*, *c* forms of *mt-3* remain always *mt-3* and similarly the *a*, *b*, *c* forms of *mt-5* remain always *mt-5*. However, a close parallelism exists between the *a*, *b*, *c* forms of the two unstable miniatures. Judg-

ing by their behavior and by their reaction to the modifiers of mutability the two series appear identical. Thus the alleles at the miniature locus may be arranged in a two dimensional series as follows:

<i>mt-1</i>	<i>mt-2</i>	<i>mt-3b</i>	<i>mt-4</i>	<i>mt-5b</i>
		<i>mt-3a</i>		<i>mt-5a</i>
		<i>mt-3c</i>		<i>mt-5c</i>

The changes responsible for *a*, *b*, *c* forms (secondary changes) may be connected with the chemical reactions which are responsible for reversions to wild type (primary changes), or they may be entirely apart from them. In the latter case they may occur in the same gene molecule or even they may occur in a different molecule. However, whatever these changes may be, they are unstable and they are influenced by the primary changes since the increase in the frequency of primary changes increases also the frequency of secondaries.

The above diagram of the unstable alleles at the miniature locus may be expanded as follows to in-



clude the secondary changes. In this diagram the sections dealing with the changes to wild type are omitted since they are identical with the sections shown in the previous diagram. If they were included the diagram would have four parts, that is, two for each section shown here. The left side of the diagram pictures secondary changes in two unstable miniatures as independent of each other, and the right side of the diagram pictures the secondary changes independent of the unstable miniature changes which may be located in the same or in a different gene molecule.

Before concluding this review of the situation found in unstable genes of *D. virilis*, I wish to say a few words about cytological studies made on chromosomes of this material. A few geneticists are still inclined to interpret the behavior of unstable genes as due to chromosomal aberrations. Although extensive linkage tests made with reddish (Demerec, 1928b) and with miniature (Demerec, 1926) make any such interpretation highly improbable, studies of salivary gland chromosomes were made to obtain farther evidence. At the time of these studies, unstable reddish had been lost already and the salivary gland chromosomes of the stable reddish derived from the unstable allele were examined. They are entirely normal. In the case of unstable miniature, salivary gland chromosomes of

the homozygous and the heterozygous individuals of *mt-3a* and *mt-3c* forms were studied. It was again found that the chromosomes are normal. This analysis was confirmed by Dr. Eileen Sutton. Therefore it seems reasonable to conclude that the changes in these unstable genes are not connected with chromosomal aberrations.

One peculiar characteristic of unstable genes is the fact that they are extremely rare in animals. Only four well established cases are on record, and all four were found in *D. virilis*. A great deal of unsuccessful effort was made to find unstable genes in *D. melanogaster* and in other animals. However, they are quite numerous in plants. The closest known parallel to unstable genes in *D. melanogaster* is provided by the so-called mottled or variegated characters obtained in irradiation experiments. As first demonstrated by Schultz (1936), these mottleds are connected with chromosomal aberrations involving heterochromatin. These mottleds occur frequently and a large number of them have been studied. In our laboratory we have a collection of 35 mottleds affecting the white locus. One important difference between them and unstable genes is that they show phenotypical changes only and do not affect the germ cells. In that respect they behave like the *mt-c* form of *D. virilis*. Since germinal changes do not occur, it is not possible to ascertain with certainty whether or not mottling is caused by changes in the gene. From the evidence available, I am inclined to believe that the mottling is caused by either the instability of the gene or by its inactivation and subsequent renewal of activity.

One of the most interesting contributions of the work with unstable genes is the discovery of genes which affect the mutability of other genes. In the case of miniature, these mutability stimulating factors act specifically on miniature alleles which are already unstable; in the case of dotted in maize (Rhoades, 1938) the mutability factor affects a specific gene which is otherwise stable; and for the mutability factor described in *D. melanogaster* (Demerec, 1937) indications are that it increases the mutability of the whole gene complex. This serves as another demonstration of the close inter-relationship between the genes of a gene system.

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DISCUSSION

STANLEY: In the case of viruses, it is known that different strains of tobacco mosaic virus which have proved to have different chemical structures can give subgroups like the *a*, *b*, *c* forms which you describe for unstable genes.

GOWEN: What is the crossover relation in *D. virilis* with respect to age?

DEMEREC: As far as I know no studies of age effect have been made on *D. virilis*.

PLOUGH: It is difficult to assume that this is a typical gene picture. Ordinary stable genes seem to have a temperature coefficient while unstable ones do not. Unstable genes are atypical in this respect and probably one should be careful in generalizing too far from this case.

DEMEREC: Probably there is no typical gene picture and I think there is no real difference between stable and unstable genes, but a graduated series from stable to unstable.

DUNN: The *a*, *b*, *c* forms might be a peculiarity of the wild type stock used. Has *mt-a* been backcrossed to different wild stocks?

DEMEREC: No, the *a*, *b*, *c* forms were obtained by inbreeding from the same stock.

DUNN: The stock came from the same male?

DEMEREC: Yes.

SCHULTZ: In the case of mutable genes in Delphinium, Dr. Demerec made the important analysis that the cells resulting from a single division might be different, one mutant, the other not. I have obtained similar evidence for the cases of variegation in *Drosophila*. This may indicate that the process of mutation is related to the division process, and indeed Haldane has made a theory of mutation on the basis of "false copying" from consideration of such cases. Are there analogous data for other cases described?

DEMEREC: I tried to obtain with *mt* data which would be comparable to those available for the unstable rose gene of Delphinium, but *mt* is not good material for such comparison, since the size of the spots is not classifiable.

STERN: Timoféeff-Ressovsky, Zimmer and Delbrück have published a discussion in which they deduce that the temperature coefficient of the mutation rate should be lower in unstable than in stable genes.

DELBRÜCK: Our argument was this: The mutation rates of the mutable genes are comparable to the rates of other normal physiological processes. Therefore we would not expect the temperature coefficients of these mutation rates to be very different from the temperature coefficients of normal physiological processes. On the other hand, the rates of the ordinary spontaneous mutations are many thousand times smaller. If they are monomolecular reactions this would mean that the activation energies of these reactions are considerably higher. It is therefore reasonable to expect that the temperature coefficients of these reactions are higher than those of the ordinary physiological processes.

The appropriateness of this argument depends on whether it is legitimate to treat the mutations as monomolecular reactions occurring in static molecules. The evidence that has been accumulated concerning the dependence of the mutation rates of the mutable genes on other genes makes me doubtful in this respect.

DEMEREC: Some unstable genes have a temperature coefficient, others do not. However, we do not know if all stable genes have it.

PLOUGH: Rhoades' case, however, gave a negative temperature coefficient, which is quite different.

GOWEN: In some bacterial forms, strains with high mutability have been studied; in these strains, the temperature effect goes both ways—low temperature does the same things as high temperature. This is an interesting field and needs more work.

BANTA: It seems remarkable that Dr. Demerec found in *D. virilis* four unstable genes in a relatively short time and that such have not been found elsewhere. Have you looked for them in other species of *Drosophila*?

DEMEREC: I have looked for many years but have found none.

INDUCED MUTATIONS IN DROSOPHILA

H. J. MULLER

1. THE QUANTITIES AND QUALITIES OF THE EXPRESSED EFFECTS

To date, radiation has stood as the only external agent by the artificial application of which mutations can be produced in abundance and with predictable frequency. The inordinate effectiveness of X-rays and related radiation in this respect has hardly been realized to the full. For when we say that a dose of 10,000 r-units results in about 100 times as many lethals per X-chromosome in *Drosophila* spermatozoa as ordinarily occur in untreated material, we do not take into consideration the fact that, in most such experiments, all these mutations were produced by a treatment lasting an hour or less, whereas the "natural" mutations represent the accumulation of a whole *Drosophila* generation, that is, of two weeks or more. When this time difference is taken into the reckoning, we find that, during the time of treatment here in question, not 100 times but at least 35,000 times as many mutations were produced in the treated as in the untreated material; moreover, by the use of the tubes of higher radiation output now available, this rate of production could be stepped up almost indefinitely.

With the above dose, every surviving major chromosome-arm would on the average have one (or somewhat more than one) *detectable* lethal or detrimental mutation. As was found by Kerkis and myself (1934, 1935, 1938) and independently by Timoféeff-Ressovsky (1934, 1935a) there are in the X-chromosome twice as many "detrimental" mutations produced—that is, those which, though not completely lethal, depress viability to such an extent as to affect the ratios appreciably in a bottle count of ordinary size—as there are completely lethal mutations. The "visible mutations" seen by ordinary methods are (in the X) a fifth to a tenth or even a twentieth as numerous as the complete lethals, depending upon the visual acumen of the observer and the degree of differential viability imposed by breeding and culture conditions. This still fails to give us anything like a full census of the mutations produced, for we miss an unknown number having effects which by our present very crude methods remain subliminal and of mutations at the other extreme of expressivity, having dominant lethal or "detrimental" effects. As in a spectrum, the "visible" portion of the mutational effects constitutes a relatively small interstitial sector of the whole range, and even the portion detected by specific techniques having greater refinement than that of ordinary inspection may still omit the greater part of the total. As for the "ultra" portion of the mutational spectrum, the existence of dom-

inant "detrimentals" incompletely lethal when heterozygous is clear from the cases in which visible effects are concomitantly produced, but no estimate has yet been made of their number, though this would, experimentally, be a quite feasible task. An example of a completely lethal dominant is a case involving a deficiency of the "bulb" of the X, found by Alikhanian in a count of 22 lethals produced in the left end of the X in a region "protected" by the presence of a duplication. However, the fact that small deficiencies for most regions have some degree of viability in heterozygous condition is, as was pointed out by Fano, Demerec and Kaufmann at this Symposium, evidence of the rarity of completely lethal dominant gene-mutations.

The conclusions thus arrived at for radiation mutations can probably be carried over to a considerable degree to spontaneous mutations as well, provided we make allowance for the greater frequency of minute deletions and other rearrangements in the irradiated material. For practically every known spontaneous mutation for which a search has been made in irradiated material has been found there, including even the various types of alleles at given loci. Thus it is probable that at least the distal end of the path of events which produces the radiation mutation is in many cases the same as that which produces the spontaneous mutation.

2. INDUCED GENE MUTATIONS AS EFFECTS OF CHANGES INITIATED IN SINGLE ATOMS

As to the mechanism of production of those radiation mutations which affect individual genes or narrowly circumscribed chromosome regions, the most important fact that has come to light is the dependence of any given mutation upon a change that was initiated in a single individual atom, by its ionization or other excitation. This is shown by the series of findings demonstrating a strictly proportional relationship between the frequency of the induced mutations and that of the induced ionizations, no matter how (within sufficiently wide limits) the latter are distributed in time and space, provided the stage treated and the conditions accompanying treatment be kept constant. The proportionality of the mutation frequency to the radiation dosage, as thus defined, when the dosage is changed by changing the duration of treatment, was shown by Oliver, Hanson and others of my earlier collaborators, by Timoféeff-Ressovsky, and by others. The inconsequentiality of the time distribution of treatment was shown by Patterson, by Timoféeff-Ressovsky, and by others. And the independence of the effect from the spacing of the excitations or ionizations, when this is varied along a range from

very soft X-rays to gamma rays, was shown by the results of Hanson, of Timoféeff-Ressovsky and his co-workers, of Fricke and Demerec, and of others, when these results are considered in connection with one another.

Despite the above series of data, many radiologists and medical men have been loth to accept the conclusion that the effect is really a single-atom one and that it is therefore, unlike most other biological and medical effects of radiation, totally lacking in any semblance of threshold value, or falling off in efficiency as very low intensities (or number of ionizations produced in a given time) are approached. As the previous studies had been carried on over a range from some 200 r down to one r per minute, my co-worker S. P. Raychaudhuri and I therefore undertook to study the mutation rate at levels of only one twentieth and one one-hundredth of one r unit per minute, distributed over one month, for comparison with the usual values. It turned out (Raychaudhuri, 1939; Muller, 1939a) that the results fell quite in line with those obtained at high intensities, and that even at these low levels the mutation frequency remained sensibly proportional to the total ionizations. In these experiments the treatment had been such as to cause the production, on the average, of only one ion-pair in a given spermatozoon in about five hours, a time which is of course inordinately longer than the life of the ion as such. To be sure, the ions are not spaced evenly so as to conform with this average value, but come in spurts. But since in this case gamma rays were used, the distance between the small groups of ions produced in an individual spurt must have been so great that they were ordinarily out of range of each others' effects, so that very few such groups would have been simultaneously produced in the given spermatozoon. Since even under these circumstances there was no falling off in the mutational effectiveness of the ionizations, it seems quite safe to conclude that the mutations start as single-atom changes.

Since the individual mutations start in single atoms it is legitimate to ask how often an excitation or ionization taking place within a given gene will be (or will happen to be) followed by a detectable mutation of it, although we must not yet assume that this mutation did really result from one of these particular changes rather than from one occurring outside of the gene affected. Moreover, in attacking this question we can only employ a figure representing maximal possible gene size, since we do not yet have a way of determining the actual gene size. Taking the figure thus arrived at by the most refined technique yet available (involving a determination of the minimal number of genetically different positions of breakage possible in a given limited chromosome region, as reported by Muller and Prokofyeva, 1934 and by Muller, 1935), and taking the highest figure reported for induced mutation rate in a given locus (that of white as re-

ported by Muller, 1928, and later confirmed as to its approximate value by Patterson, Timoféeff-Ressovsky and others), we find that a detectable mutation in this gene occurs in only one out of at least 200 cases where an ionization has taken place in the calculated maximal volume. Using the average frequency of induced mutations (including lethals and detrimental) detectable by our present techniques, instead of the frequency at the white locus, we find about a third as much chance for mutation as the above, i.e., only one mutation following at least 600 ionizations in the volume inclusive of the gene in question.

If we assumed, as some do, that nearly every ionization within the gene is followed by a detectable mutation in it or by its destruction as a gene, the above results would mean that the actual gene is hundreds of times smaller than the maximal volume above calculated. Such an assumption is however extremely unlikely. And it is also unlikely that the smaller volume thus calculated— $(.003\text{m}\mu)^3$ to $(.005\text{m}\mu)^3$ —represents a "sensitive volume," in the sense of a given group of atoms the ionization of any one of which infallibly causes the gene to be lost or to undergo some other detectable (*sic!*) mutation. The change in the calculated sensitive volume with change in the conditions or stage at which the irradiation is given shows that ionization of a given atom may on one occasion result in a mutation and not on another occasion. Moreover, the spreading of the breakage effects to more than one locus (mentioned below in the discussion of minute rearrangements) shows that it cannot be assumed that a genetic change will be confined to the molecule or gene in which the initial atomic change took place.

Nevertheless, the size of the above "sensitive volume," comprising some hundreds of atoms at least, is sufficient to prove that there are hundreds (if not many thousands) of atoms, the ionization of any one of which can result in the occurrence of a mutation of sensibly the same type. It is unlikely that this would be true (especially in the case of reverse mutations in general, as Timoféeff-Ressovsky has pointed out) unless there was a tendency for the original atom-change to initiate, by some transfer of energy, a chain of reactions so canalized as finally to result in a given, probable end-result: the mutation of the given kind.

It is important to observe that the above conception of the mechanism of gene mutation is in good agreement with that, outlined by Plough in this Symposium, which has been arrived at from a consideration of the influence of temperature (within the range normal to the organism) on the mutation rate. For in that case too the mutation is conceived as the end-result of an individual excitation, brought about however by the milder influence of thermal agitation rather than by high-energy radiation.

For the interpretation of the mechanism of in-

duction of gene mutation in certain of its other aspects, it is desirable to consider the results in this field in connection with those which have been obtained from the analysis of the mechanism of induction of cytologically visible changes in the structure of the chromosomes.

3. THE MECHANISM OF INDUCTION OF GROSS REARRANGEMENTS

It has become clear that in *Drosophila* in the vast majority of surviving cases of rearrangements (if not in all) the chromatin must have become broken at two or more points and that broken ends must have reunited with one another two by two in a new linear order. It is not implied here that restitutions, resulting from reunion in the old order, cannot occur, but that they are ordinarily indistinguishable from the original type. Where there are more than two breaks, the exchange can be a multiple exchange, of "cyclic" type, rather than a mere double exchange or combination of double exchanges as in crossing over. Whether the new arrangement is to be classified morphologically as an inversion, deletion, mutual translocation, insertional translocation, shift, or still more complicated rearrangement depends merely on the accident of where the breaks happened to be produced and which ends happened to unite with one another (Muller, 1932, 1938). A broken end facing towards the centromere, "centripetal," may unite about as readily with another centripetal broken end as with a "centrifugal" one, judging by the similar frequencies of occurrence of large deletions and inversions.

It turns out that for the induction of these rearrangements, when the breaks are far apart ("gross rearrangements"), there must have been a primary change (ionization or excitation) in more than one atom (Muller, 1936, 1938, 1939b, 1940). For, unlike what is found for gene mutations, the frequency of these rearrangements is not proportional to the number of induced ionizations, but to an exponent of the latter. In other words a second treatment, applied after or along with a given treatment, causes more mutations than if applied alone. This result shows that the effect of one primary change somehow interacts with that of another in the production of a rearrangement. The exponent of the dose as empirically determined is about 1.5 at the heavy doses (1500 to 4000 r) more frequently used, and nearer 2 at lower doses (Muller, *ibid.*). These are the values to be expected if the individual ionizations or other excitations produce single chromatin breaks and if the union of broken ends derived from different breaks takes place randomly, so far as their relation to the centromere ("centrifugal" or "centripetal") is concerned. It is easy to see that, at low doses, the chance of getting two breaks and a resulting simple type of rearrangement would vary nearly as the square of the dose, according to this mechanism. At high doses, the

exponent in question would however fall more and more below the square. This is partly because of the "saturation effect." But it is also because the multiple breakages, produced with disproportionately high frequency at higher doses, have more chance of giving, by the above type of random reunion, inviable acentric and di- and multicentric chromosomes than do the double breakages, so that more of the rearrangements produced at high doses are lost. Making allowances for these two complicating factors, it turns out that, on this interpretation, the number of actual breaks is proportional to the number of induced ionizations, i.e., the break itself is a single-atom effect, just as a gene mutation is.

That in the induction of gross rearrangements the individual atom-changes do produce individual effects, which remain independent of one another during the time of treatment of the spermatozoa, is demonstrated by experiments (Muller, 1939, 1940, confirmed by independent experiments of Catcheside, Kaufmann, Dempster and others) which show that, for a given total dose, the frequency of rearrangements produced is constant regardless of the time and space distribution of the ionizations, within very wide limits. That is, a given dose whose application is concentrated into about forty minutes has the same effectiveness as one protracted evenly over a month (and hence of less than one one-thousandth the intensity of the first) or one divided into four ten-minute periods spaced at weekly intervals; and 50 KV X-rays have the same effectiveness as gamma rays of the same ionization power. This seems at first sight contradictory to the above discussed finding that one atom-change interacts with another in producing a rearrangement, for it means that, during the time of treatment, the genetic effects of the individual atom-changes remain separated and independent. The conclusions, derived from the two series of facts considered in connection with one another, are therefore inescapable, first, that the single-atom effects do remain separate from one another during the time of treatment, i.e., during the spermatozoon stage, but second, that they enter into combination with one another *after fertilization*. The single-atom effects are evidently the individual breaks or "potential breaks" (Muller, 1940), and the combination effects are the unions of the broken ends derived from different breaks.

If ordinary cells are irradiated, in which as in the fertilized egg the unions of broken ends can presumably take place (since the chromosomes are in a similar condition in both), there should be less opportunity for broken ends to accumulate and result in rearrangements. For ends derived from the same break should in these cases more easily find one another and reconstitute the original arrangement before they become moved apart. This expectation agrees with the finding that rearrangements are induced with far greater difficulty by ir-

radiating immature germ cells than by treating spermatozoa.

That the one-atom effects induced in the spermatozoa are really the breaks, or "potential breaks," is further indicated by the results concerning losses of individual chromosomes. For on our interpretation a broken end that fails to find another broken end to unite with should occasion the loss of the broken ("ateleric") chromosome—through the mechanism reported by McClintock of sister broken chromatids becoming united to form a mitotically unmanageable dicentric chromosome. These losses, caused by single breaks, should—unlike the gross rearrangements above discussed—be nearly proportional to the number of ionizations, at ordinary doses (the exponent should in fact fall gradually below 1 at higher doses because of the greater chance for rearrangements at the expense of losses at higher doses). That this linear relation does hold for induced chromosome losses has been shown in recent experiments by the author and Pontecorvo (Muller, 1940; Pontecorvo, 1941). Moreover, we have obtained data, as yet unpublished, which give evidence from another angle that these losses are really due to breakage. These data show that in ring chromosomes, in which breakage would, for structural reasons, be especially likely to result in loss rather than perfect restitution, the obtained frequency of the losses is in fact higher. All this supports both the conclusion that the breaks are one-atom effects and the conclusion that union occurs a considerable time after breakage, when spermatozoa are treated.

Our results from sex-ratio studies on the ring and ordinary chromosomes, following Bauer's (1939) method, further show that not more than about a seventh of the individual chromosome losses are actually detected. The rest, the "lost losses," somehow result in the death of the zygote. This is a phenomenon similar to that which we (Pontecorvo and Muller, 1941) had previously described for dicentric chromosomes resulting from translocations. These inviable losses give rise to a considerable effect on the sex ratio and "dominant lethal" effect, of a measurable kind (which has not hitherto been taken into account sufficiently in studies of the infertility of treated males). Calculating back from these results, it turns out that, at the doses usually used, there are several times as many breaks which in ordinary chromosomes are followed by restitution (but many of which, in the case of ring chromosomes, are lost by axial rotation of parts before their reunion and so cause measurable loss and lethal effects) than breaks which result in either gross rearrangements or individual chromosome losses. This high frequency of restituted breaks, in proportion to rearrangements, explains why the exponent of the dosage to which the frequency of chromosome losses is proportional remains so nearly 1 at ordinary doses, rather than showing that diminution of its value, as the dose

rises, which would reflect the lesser availability of broken ends for producing individual chromosome losses at higher doses, occasioned by their greater likelihood of forming gross rearrangements. And it gives us pause for thought when we consider that even at ordinary doses there has been more than one break per gamete, but that the break has usually been an invisible, "restituted" one. How often may this "restitution" have been imperfect or attended by some local alteration such as a "gene mutation"? Another consideration arising from the finding that the frequency of restitutions is so much higher than that of rearrangements or losses concerns itself with the mechanism of rearrangement in general. For the greater frequency of restitutions than of rearrangements in this material can only mean that, even after the treatment of spermatozoa, the original propinquity of the parts to one another plays an important role in determining the likelihood of the union of their broken ends. In this sense, then, the unions are not at random, even after spermatozoon irradiation.

Several other kinds of evidence of this propinquity effect have been reported. For one thing, there is the finding, in work by Sidky and myself (Muller, Makki and Sidky, 1938; Muller, 1940), that insertional translocations are probably more frequent, in relation to two-break translocations, than random union would allow. This fact we explained on the basis that, when there were three breaks, the union of a broken end derived from one break with another derived from another break, by depriving the complementary ends of the chance for restitutional union with the ends that were originally most nearly adjacent to them, made them more likely to unite with ends derived from a third break than if the propinquitous ends had remained available. Secondly, there is the finding reported by Bauer, Demerec and Kaufmann that two broken ends derived from two different breaks in the same chromosome arm are more likely to unite with one another, giving inversions, than are the broken ends from two breaks in different chromosomes, giving translocations, although it is surprising that, this being the case, the occurrence of shorter inversions and deletions does not seem to be preferred to longer ones. Thirdly, there is their finding that in the occurrence of rearrangements involving four breaks two separate "double exchanges" occur significantly oftener, in relation to the expectation for random union, than "cyclic" exchanges of the ends from all four breaks.

A possible fourth mode of expression of the tendency to preferential union of nearby breaks is furnished by the comparative rarity, even at high doses, of cases of insertional translocation or of shift (like that of scute-19,—see section on minute rearrangements), in which a minute interstitial region, excised by the occurrence of two closely neighboring breaks, becomes inserted at some third point of breakage, far removed from these. Random

reunion would cause such cases to be rather common, judging by the frequency of minute deficiencies and of breakage in general. Their rarity, in comparison with cases of insertion of larger regions, has been indicated in studies on ring chromosome translocations carried out by Sidky and the author. The interpretation that at first seems more plausible is that the two ends of the tiny excised piece, lying so close together, are much more likely to unite with one another to form a small acentric ring, that is lost, than to unite with the ends derived from a distant break. However, as we shall see in the section on minute rearrangements, the reputed production of these, without a corresponding number of gross rearrangements, by ultraviolet light, raises the question of whether or not they are usually produced by essentially the same sort of chromosome breakage and reunion: if they are not, the above conclusion would not follow here.

No matter how the question just discussed may be decided, however, there can at present be little room for doubt, on taking all the above lines of evidence together, that broken ends of spermatozoon chromosomes which are nearer together when they enter the egg have on the whole more chance to become united than those which are further apart. When the irradiation is applied to the expanded nuclei of ordinary resting cells, moreover, it is to be expected that the tendency to propinquitous union would be far stronger than when applied to spermatozoa. For in resting nuclei: (1) there is much less opportunity for migration of chromosomes and chromosome parts, (2) the union can go on during treatment, making ends derived from earlier breaks less available for union with those from later breaks, and (3) perhaps the distance between broken ends belonging to different chromosomes may be effectively greater, as compared with that between ends from different breaks in the same chromosomes, than is the case in the nuclei of the fertilized egg.

There is good evidence that at least the first two of these factors are operative when resting plant cells are irradiated. In harmony with this conception in *Drosophila* is the empirical result that the ratio of induced rearrangements to gene mutations is considerably lower in immature germ cells than in spermatozoa (Muller, 1930) and that, in fact, as Glass (1939a, and this Symposium) has reported, it is extremely difficult to produce translocations at all in these stages, though more inversions occur when mature eggs are treated. The latter observation probably results from the broken ends being unfusible while the chromosomes are in the condensed stages of metaphase and anaphase, just as they are in the spermatozoon, so that breaks accumulate; in the stages following, the different chromosomes are evidently held apart from one another long enough, during the period in which broken ends can fuse, to allow those derived from the same chromosome to unite before those derived from different chromosomes can. In further agree-

ment with the conclusion that propinquitous broken ends usually undergo restitution in ordinary cells before there is opportunity for them to come into contact with ends derived from other breaks are the data which have been reported on genetic changes induced by irradiation of embryonic and larval somatic cells. For when reexamined these data show little or no sign of the production of deletions or of terminal deficiencies in the soma, the phenomena described under this heading giving every indication of being in the majority of cases due to somatic crossing over.

Whether in addition to such preferential union there is also preferential breakage of different regions of the chromatin is a debatable question. Certainly there is *in effect* preferential breakage. For, as has long been known, the heterochromatic regions near the centromere (and the same regions even after having been removed from the neighborhood of the centromere, by some prior rearrangement) show far more rearrangements, in proportion to their number of contained genes or length of salivary chromosome (or of mitotic chromosome, when due allowance is made for the blocks), than do the euchromatic regions. And the same is true, to a lesser degree, for the heterochromatic regions near the telomeres. As Prokofyeva-Belgovskaya (1939) and Kaufmann (1939) have recently shown independently, by exact breakage counts, the partially heterochromatic regions that they have found to lie interspersed in various interstitial positions show a similar tendency. Since however we know that union can to a certain extent be preferential we cannot without further evidence know to what extent the indisputable regional differences in frequency of rearrangements represent differences in the frequency with which the breaks actually occurred, rather than differences in the frequency with which, after breakage, the ends tended to undergo restitution in preference to recombinational union. For instance, the two opposite ends derived from a breakage in a heterochromatic region might have more of a tendency to become drawn apart, and so later to undergo rearrangement instead of restitution, than those in a euchromatic region. Another way of describing this possibility might be to say that in the heterochromatic regions more of the potential breaks became actual than in a euchromatic region. While preferential breakage as such is not inherently unlikely, the answer to this question of the mechanism involved in the production of the observed differences in rearrangement frequency must await some new kind of evidence.

4. THE QUESTION OF TERMINAL DEFICIENCIES

To what extent are we justified in holding to the conception that in *Drosophila* a broken end will always seek to unite with another broken end rather than becoming "healed," and that in consequence the telomere is a permanent chromosomal organ? In 1932 I called into question the validity of the

assumption that "terminal deficiencies" were possible in *Drosophila*, and both before and after that a group of my collaborators and I, including Belgovsky, Prokofyeva, Raffel and others, have been accumulating and reporting evidence on the subject, although the assertion has been made that evidence is lacking here.

First, a rather extensive series of counts, using yellow and scute as markers, failed to reveal an apparent case of loss of a piece of the X broken between yellow and Patterson's "viability gene," though there must have been a good many breaks in this region that were followed by rearrangement (Muller, 1932). The one case (scute-J4) in this series that seemed on salivary examination to show a terminal deficiency of this region had the terminal piece in question apparently attached to the left end of the third chromosome, so unless this case included the double "miracle" of having a telomere converted into an interstitial gene as well as *vice versa*, a minute end-section of III had in fact been exchanged for the scute-bearing region of the X. No doubt in a more extensive count a still better simulacrum of terminal deficiency would be found, but that is to be expected as a result of breakage with rearrangement occurring disproportionately often in the terminal heterochromatic region, since our other results have shown a single gene, especially in that region, not to be demonstrable with certainty by ordinary salivary technique.

It was then found that if a piece of heterochromatic region derived from the neighborhood of the centromere had been placed close to the right of the region bearing the scute locus, as in scute-8 and scute-L1, cases which seemed from preliminary genetic analysis to be terminal deficiencies of this region occurred much oftener. But salivary as well as further genetic analysis showed these still to possess the terminal parts; that is, they were cases of minute rearrangement, either of the inversion or of the deletion type (Muller, Prokofyeva-Belgovskaya and Raffel, 1937; Belgovsky and Muller, 1937; Raffel, 1938). Taking advantage of this fact that effective breaks occur more readily in heterochromatic regions, we then made use of an X-chromosome containing the Bar-M2 inversion. In this I had found one of the breaks to be just to the left of the normal allele of Bar and the other break close to the left of the centromere. X-rayed Bar-M2 males (4000 r) were crossed to females with the genes scute vermillion forked and carnation. Terminal deficiencies caused by breakage occurring either in the displaced heterochromatic region or anywhere in the euchromatic region between it and the normal allele of carnation should result in viable males with non-carnation eyes, and breakage between the carnation and Bar loci should give carnation bar-like eyes. No such males were found by Belgovsky (1938) in a count of over 14,000 flies. At the same time, however, there were, as expected, more frequent cases of females showing forked than oc-

curred when ordinary X's were irradiated. And an unusually high proportion of these forkeds were connected with lethal effects and/or mosaic manifestation, like the yellows induced in a scute-8 chromosome. This showed that breakage with rearrangement was in fact occurring with high frequency in and near the displaced heterochromatic region here concerned, even though terminal deficiency was not.

If then we take into consideration the high frequency of breaks that are followed by rearrangement, and add to these the still higher frequency of breaks followed by restitution, the absence or paucity of apparent terminal deficiencies is seen to be very significant. I would not imply that an interstitial gene of *Drosophila* can never be converted into a telomere, or *vice versa*, by a process resembling mutation, as suggested by Kossikov and myself (1935). In fact it would be strange if this process could never occur when we consider that the two types of genes were in all probability derived at some distant time from one original type, and that in sporophytic tissues of some plants at least they are at the present day convertible into one another by a "regulatory" process. But the evidence indicates that in *Drosophila* such a mutational or "physiological" change, if it occurs, must be very rare, and that, in consequence, the telomere is here to be regarded as a virtually indispensable, permanent organ of the chromosome.

5. POSITION EFFECT

An adequate treatment of structural change in chromosomes in its relation to problems of the gene must include a consideration of position effects, but this subject is too involved to be more than briefly touched upon in the limits of the present paper. It is necessary, however, to emphasize the point that the existence of the position effect in *Drosophila* is a fact admitting of no doubt. As conclusive evidence on this point I do not refer to the innumerable findings showing the comparative regularity with which genes in the neighborhood of chromosome breaks (rearrangements) exhibit changes in their functioning resembling those caused by mutations of the same genes. For these findings have been open to two alternative interpretations: firstly, that gene mutation itself tends to occur in the neighborhood of the break, as a secondary result of the same primary change which also induced the break, in the same way as a second break near the first one may also be induced simultaneously, giving a minute rearrangement; or, secondly, that the change in gene functioning comes about as a result of the fact that the breakage under consideration or some second breakage nearby occurred inside of the affected gene and thus changed it directly.

The really valid evidence for position effect may be listed under three heads: (1) In cases where a locus is known to have various alleles giving differ-

ent phenotypes, with seldom the same one twice, it is found that on the recurrence or near-recurrence of a given kind of rearrangement, bringing the gene in question into association with largely the same neighbor genes as in some previous rearrangement, the phenotypic change is of the same or very nearly the same kind in both cases; this is illustrated, for the scute locus, by scute-4, scute-L8 and scute-S1 (Muller and Raffel, 1937, Raffel and Muller, 1940), and for the Bar locus by Bar-M1 and Bar-M2 (Muller, *Drosophila* Information Service, 3:29). Influence of the type of region on the degree of expression of the mutant "cubitus" has been shown in experiments of Panshin (1935). In the same general category belongs the fact that transplantation into the neighborhood of heterochromatic regions tends to give a characteristic type of effects (the mosaicism of "eversporting displacements," as pointed out by Muller, 1935d). (2) The converse of the preceding evidence is provided by the remarkable case of Grüneberg (1937) in which the reversal of a gross rearrangement (inversion), restoring the original gene order, was accompanied by reversion to normal of the phenotypic effect ("roughest eye"). (3) The most elegant evidence of all (so far as position effects connected with induced rearrangements are concerned) is that provided by the experiments of Panshin (1936) on the curled locus and of Sidoroff and Dubinin (1935) on that of hairy. In these works it was shown that, when a normal allele derived from a chromosome of normal structure is substituted by crossing over for a gene which, since the occurrence of a rearrangement in its vicinity, has functioned like a mutant gene, the substituted normal gene now takes on the properties that the apparent "mutant" gene had, while, conversely, the removed "mutant" gene reverts to normal functioning after its transference back into the chromosome of normal structure.

The various crucial cases listed above are, to be sure, all cases in which the position effect in question was exerted by a heterochromatic region upon a euchromatic one, and it is evident that the position effect exerted by heterochromatin has certain very special and peculiar features (see Muller, 1935d, 1938, and Schultz, 1936). In the nature of the case, however, as good evidence could hardly have been expected from purely euchromatic rearrangements, because (1) breakage occurs so much less frequently in any given position in such regions that the obtaining of anything like exact recurrences or reversions is almost out of the question, (2) the position effect in euchromatic regions usually extends over a distance so much shorter, and the genes there are so diverse in their positional influence, that much more exact recurrences and reversions would often be necessary in order to secure the phenotypic effect in question; and (3) the short distance between the break and the gene affected in these regions usually removes the possibility of the crossover test.

It may be recalled, however, that the original case in which position effect was discovered—that of Bar, found by Sturtevant, (1925, 1928)—is a truly euchromatic rearrangement, which happens to extend over a longer distance than usual for such cases, and that in this very case the critical test of crossing over succeeded. And although we do not have anything like exact cases of recurrence of euchromatic rearrangements, evidence of essentially this kind is provided at the scute locus, for instance, by the observation that no euchromatic rearrangements affecting scute are known in which the break near scute is to the left of it though there are several cases of breaks close to the left that do not affect scute, while on the other hand there are many known euchromatic rearrangements affecting scute in which the break is to the right of the scute locus. Evidently this is because a certain gene or genes normally to the right of scute exerts a much stronger effect on the functioning of this locus than any gene to the left of it. It is of interest to note further that in these euchromatic rearrangements involving breaks to the right of scute, which are by the evidence here mentioned inferred to be position effects, the break has been shown to be not merely to the right of scute but even to the right of the gene (whose homozygous deficiency acts lethally) which itself is just to the right of scute. That is, the break was at least one gene removed from scute. Hence too it is very unlikely that there was in these cases a break inside of the scute gene itself. (Even if it were assumed that there had been a double break in these cases, one within and the other outside of scute, there would be no apparent reason why the break outside should always be to the right of it.) All in all, then, there can be no reasonable doubt of the reality of purely euchromatic position effects as well as of those involving eu-heterochromatic substitutions. And it is very likely, from analogy with the many analyzed cases of gross rearrangement of scute and of certain other loci, that the great majority of apparent mutations accompanying rearrangements are really position effects.

We cannot pause here to assess adequately the relative probabilities of the different possible interpretations of the mechanism of the position effect. That it represents the direct chemical influence upon one part of a large molecule, caused by a change in another part of it, like the effect produced on a fatty acid molecule by the lopping off of a terminal methyl group, is rendered extremely unlikely or impossible by the vastness, on a chemical scale of magnitudes, of the distances involved. The interpretation as an effect of local interaction of gene products has a higher plausibility. In some ways more attractive is the possibility which I proposed in 1935 that the effect is an expression of the same forces which, in the case of homologous genes, bring about their synaptic association. Such forces, acting between unlike genes, should tend to

deform them, and this deformation might well affect the nature and the quantity of the gene products which they form. If so, position effects might appear more strongly expressed in organisms in which the synaptic forces were able to exert themselves relatively intensely in the somatic cells. This would agree with the finding of position effects in *Diptera* and their absence or paucity in maize and (as may be deduced from the work of Snell and of P. Hertwig) in mice.¹ This, however, is a field in which as yet speculation has outrun evidence.

The cause of the somatic mosaicism and other peculiarities of the position effects exerted by heterochromatic regions is a field in which speculation as well as experiment are both active at present. The general phenomenon of "eversporting displacement," in which a gross rearrangement is accompanied by the mosaic mutant expression of genes near the breakage points, was discovered early in the X-ray mutation work (Muller, 1930b), and it was also observed that some individuals showed a genetically conditioned partial suppression of the condition. That the mosaicism is not a result of mere loss of a gene or chromosome-section from somatic cells was inferred (Muller, *ibid.*) from the manner of its expression. For it was noted that in some of these cases—where the darker ommatidia of "variegated" or "mottled" eyes appear individually against a light background—the presence-absence interpretation would necessitate the assumption of a gain rather than a loss having occurred. Evidently something more peculiar than mere loss, something connected with the general mechanism of position effects, was involved. In 1933 Gowen and Gay showed that the presence of an extra Y chromosome tended to lessen the variegated mutant effect, allowing the affected genes to function more normally and uniformly. And work in the succeeding years, especially by various Russian geneticists, showed that the same normalizing effect was exerted by various parts of the heterochromatin (Y^a , Y^1 , parts of XI, etc.). As I noted in 1935 and Schultz in 1936, the mosaically expressed rearrangements always involve a transfer of the affected gene or genes into the neighborhood of a heterochromatic region; that is, the variegation is a kind of position effect peculiar to heterochromatin. Thus it was somewhat strange to find that the *addition* of heterochromatic material to the cell allows the affected gene now to become more normal in its functioning.

In the attack on this question, it was observed by Prokofyeva and myself, and independently by Schultz, that the euchromatic regions which have been transferred into the vicinity of heterochromatic ones are themselves partly, but variably, trans-

formed into a heterochromatic condition—"heterochromatized." This is shown not only by their appearance in salivary chromosomes but also (Muller, et al. 1937) by their (effective) breakability when irradiated. Paradoxically enough, when extra heterochromatin (e.g. an extra Y) is added to a cell, the heterochromatization, as seen in salivary chromosomes, is lessened; i.e., the transferred region remains more euchromatic, in correspondence with the greater normality of its functioning noted in the preceding paragraph. It is tempting to think of this influence as due to an effect of the heterologously synapsing chromatin that has been added, upon the synaptic associations of the remainder. However that may be, it is evident that the degree of heterochromaticity attained is to some extent an index of the intensity of the effect on gene functioning (usually lessening this functioning). And whatever it is that causes the heterochromaticity to vary somatically, i.e. to be more or less mosaically expressed—a problem as yet unsolved though it may have its basis in accidents of chromosome association—must also cause the gene expression to vary correlatively.

There are many characteristics which distinguish hetero- from eu-chromatic regions—among others, the pattern of association of the chromonemas, the distinctness of the bands, non-homologous association, effective breakability by radiation. Among all these known factors, it seems difficult to discover which of them are the more primary ones, and which are more closely related to the effects on gene functioning. It is believed by Schultz that these special position effects of heterochromatic regions are caused by differences in its nucleic acid synthesis, which may even go so far as to result in non-reproduction of a gene or chromosome-section. This possibility is an intriguing one, and might lead us far. But before trying it as a working hypothesis it may be well to recognize that there may be other possible causative factors, of—so far as we know—a very varied nature, and that we know very little as yet concerning the mechanism of functioning of a gene. It is a big step to see that its change of function is connected with a visible change in the appearance of the chromosome; it would be a far bigger one if we could jump from there to the chemical basis of the change.

6. MINUTE REARRANGEMENTS IN RELATION TO OTHER KINDS OF MUTATIONS

Serebrovsky in 1929 had urged that "gene mutations" might all of them be nothing more than minute deletions and other rearrangements, of fundamentally the same kind as the gross ones. And though the evidence from reverse mutations and from certain other sources raised certain objections to such a view (Muller, 1928; Patterson and Muller, 1930), it was, as pointed out by Stadler (1932) very difficult to get crucial evidence for or against it, for even reversibility may be possible in

¹ The possible significance of the occurrence of both somatic pairing and position effect in *Diptera*, combined with the absence of both in maize, in relation to the synaptic force hypothesis of the position effect, has been independently noted by Schultz (personal communication).

the case of a rearrangement. As yet, however, the occurrence of minute rearrangements (aside from small "deficiencies," of unknown mechanism of origin) was only a matter of conjecture. And while it seemed not unlikely that they might occur occasionally as extreme examples, in the direction of smallness, of ordinary rearrangements, there seemed no reason to assume that such "extreme accidents" would not be correspondingly improbable.

The genetic analysis of scute-19 (Muller, 1932-34, published in 1935b) provided the first concrete case of a minute rearrangement in which it was clearly demonstrated that two breaks very close together (probably including between them only four genes) must have occurred. Not only had the minute interstitial section in question been deleted from the X but it had been inserted elsewhere (into the second). This meant that the deficiency in the X had not been caused by the destruction of the genes located there but by the breakage and rearrangement of gene connections. A plausible explanation was thus provided for all cases of small interstitial deficiencies. In this case there was also seen to be an accompanying change in the gene functioning of the scute locus, although this locus was not immediately adjacent to either of the breaks. Here then was a very probable case of a position effect, accompanying the minute rearrangement.

It the above interpretation could be true in the scute-19 case of insertion, in which three breaks had occurred, it could also be true in two-break cases in which the interstitial section had not been lost, i.e. in inversions and duplications, which ought to occur much oftener than insertions. Such minute inversions, when they had position-effects, would pass the genetic tests for gene-mutation unless the position effects appeared to involve more than one locus. Only the scute region provided cases in which a sufficiently intensive genetic study of these matters had been made. In this region I had found that two cases, scute-J1 and "scute-10" (achaete-2), although they did not involve gross rearrangement nevertheless showed effects on more than one locus, as shown by the fact that the minute scute-19 insertion "covered" one of the effects but not the other. The salivary chromosomes of these two cases were therefore investigated intensively by Raffel and myself, and it was found (Muller, Prokofyeva and Raffel, 1934, 1935) that both of them did in fact involve minute inversions. A number of minute inversions of similar size have since been reported. (Kerkis, Patau, Horton) to differentiate *D. simulans* from *melanogaster*, and minute duplications, both "tandem" and "reflexed," have been shown to characterize normal chromosomes as well as to have arisen in irradiated material (Bar, Hairy-wing, etc.)

As the scute cases showed, the minute rearrangements must be far more frequent than is to be expected on the basis of their constituting merely the extreme variants, in the direction of smallness, of rearrangements randomly distributed as regards

size. That is, we have the right to speak of them as a special category, in distinction to the gross rearrangements, even though some intergrades are to be expected too. The recent studies of Demerec and his co-workers on deficiencies in the Notch region now give abundant quantitative proof of this conception, showing that besides the deficiencies that are randomly distributed in size there is another group, the minute deficiencies, having a size distribution of its own with its upper limit at about 15 bands. Conceivably, then, minute rearrangements might be numerous enough to "explain away" the phenomena known as gene mutations.

The minute rearrangements of size no greater than the scute-19 deficiency would, when inversions, seldom be detectible in the salivary chromosomes. These cases, when found genetically, would usually pass as gene mutations, since, as may be inferred from studies on gross rearrangements, the entailed position effects in such cases would seldom sensibly influence more than one gene (and most often would not affect any). Deficiencies of such size could usually be detected genetically as well as cytologically with much greater ease, and it is now well known (Mackensen and Painter, Demerec, Alikhanian, Sacharov, Slizynski, etc.) that such deficiencies are in fact produced in abundance by irradiation, forming a considerable proportion of the lethals. If only one or two genes were removed, however, such deficiencies would usually escape cytological detection, and some of them could appear genetically as viable visible mutations, of "amorphic" type (Muller, 1935c; Panshin, 1938).

All the above effects, though numerous, could in no wise suffice to furnish the building blocks of evolution. For the position effect ordinarily extends over such a short distance that the number of possible changes thus producible in any given gene would be far too limited, so long as we supposed the genes themselves to be fundamentally unalterable segments, with breakage occurring only between them. If however we further supposed that breakages could occur within genes—a postulate which can be shown to involve the abandonment of most of the idea of morphologically distinct genes—we should of course expect to get a range of mutant effects much greater than if the breakages only occurred between genes, and possibly sufficient for evolutionary requirements. In that case, even the theoretical distinction between minute rearrangements and gene mutations would become an unreal one (Muller, 1937, 1940; Goldschmidt, 1937, 1938, 1940).

We seem as yet to be far from the final solution of the above problems. There are however some data which should be considered in this connection. First there are those having a bearing on the mechanism whereby minute rearrangements are induced. If apparent gene mutations represent only a special case of minute rearrangements then the frequency of these rearrangements, like that of gene mutations

and unlike that of gross rearrangements, should vary linearly with the dose of radiation. Experiments were accordingly carried out (Belgovsky, 1938; Muller, Makki and Sidky, 1938; Makki with Muller, 1940) to test this question, utilizing both the minute rearrangements occurring in the neighborhood of heterochromatic regions and those in euchromatic ones. It was found that their frequency-dosage relationship is in fact linear. This result agrees with the linear relation found for induced lethals in general, inasmuch as such a high proportion of these are really small deficiencies. It is also in harmony with the above mentioned evidence that minute and gross deficiencies belong in separate categories of frequency. But although it thus leaves open the door for an identification of gene mutations with minute rearrangements, the evidence which it gives on this point is only of a negative character—it simply fails to show a difference between them. We cannot legitimately infer from it that they are the same, unless no other grounds for differentiating between them exist.

To explain the above linearity, it is necessary to conceive of the whole alteration involved in any given minute rearrangement as having resulted from a single atom-change (ionization or other excitation). That is, the two breaks included in it must both trace their occurrence to this original event. The change in this atom, then, must somehow have had effects which spread out to these two different points. These points were to be sure near together in terms of chromosomal distances, and might have been a good deal nearer than this in space if at the time of the irradiation they were lying on adjacent rungs of the chromonemal spiral (Muller, Prokofyeva and Raffel, 1935; Sax, 1938). Nevertheless they must have been so far apart, in terms of atomic distances, as to have required some special type of transfer of energy or chain reaction, in order to enable the final effect to take place at such a distance from the initial one. We do not know whether or not this process of transfer is related to the previously inferred "canalized" chain of reactions that occur in the induction of gene mutations. But it does support our contention that a primary atom-change occurring *outside* of a given gene or separable portion of the chromosome may lead to permanent changes in the latter—a conclusion which, as previously noted, raises serious difficulties with the application of the "sensitive volume" idea to the genetic material.

One conceivable method of attack on the question of whether minute rearrangements and gene mutations are essentially different is to try different methods of inducing them, in the attempt to find an agent that will discriminatively induce one type without the other. Evidence has been obtained (Altenburg, 1930, 1936; Muller and Mackenzie, 1939) that in *Drosophila*, as in plants (Stadler and Uber, 1938, and others) ultraviolet light induces apparent gene mutations, but, as compared with

X-rays of the same gene mutation-producing strength, far fewer, if any, gross rearrangements. This result seemed to make the conclusion plausible that in *Drosophila* ultraviolet, unlike X-rays, did not ordinarily break the chromosomes, at least not in any thorough-going way, and that the ultraviolet mutations were accordingly intragenic changes—a conception which in turn is in harmony with that of the existence of genes as definite segments of the chromosomes. Preliminary tests on the induction of minute rearrangements (Mackenzie and Muller, 1940), using genetic methods of detection, failed to disclose any and so it began to look as if these interpretations might receive experimental support. However, as yet unpublished salivary examinations by Slizynski, carried out during the last year on the lethals which had been induced in the above experiments by our ultraviolet treatments, seem to show that they include a considerable proportion of minute rearrangements. I do not wish to anticipate Slizynski's own account by giving details, but he would doubtless not be averse to having this result mentioned tentatively here, as it would have high relevance to the questions under discussion. In the meantime, we are continuing work along this line, in the hope of obtaining further data, and it would hardly be fair, as yet, to discuss the implications at length. It is obvious however that, if the result is established, we must admit that one more possible means of distinguishing between minute rearrangements and gene mutations has failed, while at the same time very interesting questions are raised as to how the minute rearrangements are produced without causing a great number of chromosome breaks of a type that can also enter into gross rearrangements.²

Another possible means of discriminating between the two phenomena might lie in the study of "fractional" mutants. In the case of gross rearrangements, we have interpreted these as caused by delay of union until after chromatid formation, followed by the occurrence of restitution in one of the two chromatids and of rearrangement in the other. Until the last mentioned ultraviolet data were obtained, we assumed that fractional minute rearrangements were caused in the same way. But fractional gene mutations would involve a different process, unless these too are really structural rearrangements of essentially the same though still more minute type. In view of these considerations it is of high interest to note that fractionals of all three types are in fact produced by irradiation of sper-

²In this connection it may be noted that we did find one translocation (between X and II) in ultraviolet treated material in experiments carried on at Amherst during the past year. This was detected as a sex-linked semi-lethal, in experiments in which only sex-linked lethals and semi-lethals were being looked for. Cytogenetic studies of these lethals, by P. N. Bridges and the author, do not indicate a significant production of minute rearrangements, but these observations are still provisional.

matozoa, and that although few exact studies allowing an accurate comparison have been made, the fractionals appear on preliminary inspection to occur in all three kinds of cases with similar frequency, in proportion to the non-fractionals. If this result should be found to hold and if our interpretation of the origination of fractionals in the case of gross rearrangements is correct also, we should be led to infer that the gene mutations too probably arise by some process of breakage and reunion of the chromonema.

So long as we held to the conception of the genes being strictly delimited from one another morphologically, the postulated breaks whereby "gene mutations" originated would be intragenic but the breaks of minute rearrangements would in all probability differ from them in being only intergenic. For if the latter also were postulated to be intragenic a contradiction would be involved. Not only would the genes, in the course of evolution, have in this case become greatly scrambled, so that the chromosome would have lost any original regularity of segmentation, but there would remain no good reason for supposing a segmentation, on the relatively gross scale that has been attributed to genes, to exist at all. For since the breakage points of the chromonema (at least as expressed in crossing over) have, theoretically at least, been the major features marking the genes' boundaries, the obliteration of these as distinct breakage points would tend to result in the obliteration of the concept of the gene as a distinct, individual body. On the other hand, if we supposed the concept of the individual gene to be only a proximate one, roughly describing, for our convenience, certain chromosome regions having to do with given functions, then there would be no need for postulating a sharp difference between the breaks of gene mutations and those of minute rearrangements.

The above discussion leaves out of account, however, some major considerations connected with the question at issue. Certainly the chromonema cannot be a continuum in any ultimate sense, as it is made up of atoms and these in turn are bound together to form amino-acid units. Not only do the facts already known about protein structure in general, and about the structure of nucleoproteins in particular (as disclosed for example by the X-ray diffraction studies on tobacco mosaic virus) indicate that the amino units again are grouped into higher units, in a kind of ascending hierarchy, but there are facts from the study of chromosome breakage itself, or at least "gross" breakage, to indicate that this breakage does not ordinarily occur between the smaller units, i.e., between those connected by peptide bonds. For the breakage of a peptide bond, like that of the great majority of other chemical bonds, necessarily results in fragments of opposite sign, either of which in uniting again can only unite with another of sign opposite to itself. If it could react otherwise it would form a new type of

bond, and would no longer conform to the peptide formula. On the other hand, in the breakage of chromosomes this condition does not hold. That in this kind of breakage the two fragments that unite may have in effect the same sign is shown by the fact that when such a fragment happens to give rise to its daughter chromatids before union of broken ends occurs, the like broken ends of the sister chromatid fragments can then unite with each other. Since now a protein molecule can, so far as we know, not be broken into two parts without the breaking of a peptide bond—at least on any commonly held theories of these molecules—it becomes very probable that our gross chromosome-breaks, at least, are cutting between these huge molecules rather than within them.

The same conclusion follows from the occurrence of inversions, inasmuch as there is no way of producing an inversion within the limits of a polypeptide chain or ring—no matter how this may secondarily have been folded and tied together—without destroying its polypeptide character by forming foreign units in place of at least two of the original amino-acid units. Hence there must be larger groupings in the chromonema than the amino-acid units, and inversion breakage, at least, must take place only between these larger groupings.

Another consideration pointing in the same direction is derived from the facts of crossing over. The comparative regularity of the phenomenon of interference and the dependence of its intensity upon distance as such shows plainly that mechanical stress plays a considerable part in the breaking of the chromonema that the process of crossing over entails. It is hardly to be expected that such stress would be sufficient to break a peptide bond. Moreover, it is going rather far to postulate that the chromonemata can be so extremely extended and so very precisely apposed at this time that even the individual amino acids lie exactly paired, so as to result in "equal crossing over" between them. The theory is less strained by presuming that larger structures are involved here.

So far as the genetic evidence can take us, it points to the conclusion that, in the scute and brown regions (Muller and Prokofyeva, 1934; Raffel and Muller, 1940; Glass, 1939b)—the only regions so far analyzed with reference to this question—the breaks of gross rearrangements occur in definite positions, as far apart as genes are ordinarily supposed to be. We can escape this conclusion by assuming that the chromosome regions lying between seemingly but not actually identical positions of breakage are dispensable (thus having caused the "left-right" test to fail). Not merely must they be dispensable but it must be supposed that under the conditions of the experiment these regions (presumably weakened in their functioning anyhow by the existing position effects of heterochromatin) produce by their absence no detected disturbance in the phenotypes. This as-

sumption is by no means impossible, but it serves to strain our interpretation. For the effects found in the cases where the breaks were adjudged to be really in different positions were themselves quite distinct lethal or visible effects. If the breaks did not occur in definitely delimited positions we might have expected more of the effects to be indefinite, intergrading with the cases of no effect where we adjudged the breaks to be in the same positions.

Similar evidence along this line may be seen in the fact that, in all the analyzed cases of breakage in these two regions, including also the minute rearrangements, none of the genes at or near the break seemed to have been really destroyed. It had often been changed, and weakened in its functioning, but at least a rudiment of its characteristic functioning remained. If however the break had cut through near the middle of an actual molecule we should ordinarily have expected the mode of action of that molecule to be completely altered.

It may be contended that the above are all mere straws in the wind. But they all point the same way. On the other hand, the facts indicating a relationship between gene mutations and minute rearrangements seem to a certain extent to point the other way. This indicates that the underlying facts may be more complicated than we have imagined, and that much more work is in order.

7. OTHER AGENTS THAN RADIATION

It would be one-sided to close without some reference to other possible modes of induction of mutations than radiation. The high lability of the "spontaneous" mutation frequency even under conditions normal to the organism indicates that other means of strongly influencing the mutation process must exist. But although many very drastic treatments (killing the great majority of the organisms treated) have been tried, none so far reported has met with marked success and has been retested and had its efficacy confirmed by independent workers.

The most recently reported experiments in which mutations were thought to have been induced in high frequency by an external agent other than radiation are those of Gershenson (1939), an investigator experienced in the *Drosophila* mutation technique. He raised flies upon food to which a large amount (5 percent) of thymonucleic acid had been added, and found that a considerable proportion of the imagos derived from the larvae that had had this food showed various characteristic phenotypic abnormalities. When these flies were bred, similar abnormalities were often noted among their descendants, and so it was concluded that mutations had been induced *en masse*.

I have repeated this feeding procedure, using both thymo- and yeast-nucleic acid, as well as controls. There was no doubt of the induction of the phenotypic abnormalities in the flies that had themselves got the thymonucleic acid while in the larval stage. The yeast nucleic acid produced much

lesser, if any, results of this kind, although the difference might have been connected with the fact that the food for some reason had a different consistency. The controls too showed very few, and these only slightly, abnormal flies. The flies that had emerged were then tested for the frequency of sex-linked lethals that had originated in their germ cells, by breeding their offspring (as well as themselves) in individual cultures, suitable markers with inversions having been provided. In the prosecution of this work, the nucleic acid feeding was continued, during their life time, with the adult stages of the flies that had been treated as larvae. No lethals were found in the treated flies themselves, but this was hardly to have been expected in any case. The results from the tests of F_1 were as follows:

	tested chromosomes	lethals
thymonucleic acid	520	0
yeast nucleic acid	350	0
controls	270	0

It is hardly conceivable that a variety of types of visible mutations could be induced *en masse* by any agent without there being some readily appreciable effect upon the lethal mutation frequency also. The above results are sufficient to show that there is no such effect in our material. It was not surprising therefore to find that, when the abnormal flies were bred, their peculiarities did not show any significant evidence of being inherited by the later generations derived from them, when these were not themselves raised in the same way. Of course some direct transmission of the substance fed or of its derivatives, through the egg, might have occurred in such a case, without being indicative of a true inheritance, as reported by Medvedev and others for the "vermillion" substance, but the effects of this would rapidly die out after the first filial generation and so could readily be distinguished from gene changes. No distinct effects of this kind were noted in our experiment.

There were however occasional reappearances of flies showing slight degrees of expression of some of the abnormalities in question in later generations of both the control and the treated series. This is obviously because such deviations as a slight tendency to irregular venation or roughened eyes are not highly unusual even in untreated material, and show more tendency to be expressed in some "normal" lines than in others. At the same time the inbreeding accompanying tests of this kind, by increasing the genetic diversity and the chance for homozygosis, gives a greater opportunity for the appearance of these peculiarities in some of the individuals. Possibly too the directly treated individuals which show the abnormalities most clearly are on the whole those which happened to have inherited a genotype most predisposed to favor its

expression, so that by breeding these individuals we might be practicing a more efficient selection for the type in question than when breeding the controls. However that may be, there was absolutely no indication, in our results, of either visible or lethal mutations having been induced by the treatment.

We are not presenting the above negative results as an argument that mutations cannot be induced by chemical treatment. They only serve to indicate what strict precautions must be observed in any such work, especially where visible mutations of types not previously specified are used as the only criterion. In view of the high protection ordinarily afforded the genes by the cell which carries them, and which seems determined to guard them even to the death, it is not to be expected that chemicals drastically affecting the mutation process while leaving the cell viable will readily be found by our rather hit-and-miss methods. But the search for such agents, as well as the study of the milder, "physiological" influences that may affect the mutation process, must continue, in the expectation that it still has great possibilities before it for the furtherance both of our understanding and of our control over the events within the gene. For if random mutation can introduce into the cell conditions like those found for instance by Beadle in the case of the "sticky" gene in maize, which inordinately raises the frequency of mutations of all kinds, or by Rhoades in the case of the *Dt* gene, which results in a specific type of mutation in another locus, it should eventually be possible to find artificial treatments with effects of a similar nature.

SUMMARY

1. The work on the induction of gene mutations in *Drosophila* by high energy radiation is reviewed, with particular attention to the evidence showing that these mutations start as changes in individual atoms, in consequence of which by a canalized transfer of energy or chain of reactions the mutation in the gene finally ensues.

2. The evidence from *Drosophila* is summarized showing that in the induction of gross rearrangements of chromosome parts the radiation again acts through single-atom changes, which in this case, through an analogous chain of reactions, result in breaks of the chromonema. Gross rearrangements follow when two or more breaks have been produced independently and the broken ends derived from the different breaks find and fuse with each other. Such fusion cannot occur during the spermatozoon stage, and probably not during other stages of extreme condensation of chromosomes (metaphase and anaphase).

3. If fusion has not occurred before chromatid formation, two sister fragments can unite together, with subsequent loss of the resulting dicentric and acentric chromosomes. In most cases, however, the

presence of such chromosomes in the fertilized egg results in the death of the zygote.

4. Even if the breaks have been induced in the spermatozoon stage, so that fusion is long delayed, there is a distinct tendency for broken ends which originally lay closer together to fuse with one another rather than with more distant broken ends. This favors restitution as opposed to rearrangement and chromosome loss. When cells are irradiated in ordinary resting stages this tendency is much greater. Evidence of Pontecorvo and the author shows that even at high doses the great majority of breaks induced by sperm irradiation is followed by restitution, so that the number of breaks actually produced is much greater than the number ordinarily detected or reckoned with.

5. Evidence is reviewed which shows that in *Drosophila* chromonemata once broken very seldom if ever "heal" except by the union of broken ends with one another; *vice versa*, free ends seldom if ever become interstitial. Hence the gene at the free end of a chromosome must be regarded as forming a permanent distinctive organelle, the "telomere."

6. The conclusive character of the evidence for the existence of the position effect is explained, and possible interpretations of the nature of this effect and of its existence only in certain kinds of organisms are briefly pointed out. Peculiarities of the position effect exerted by heterochromatic regions are discussed.

7. The evidence on which the concept of minute rearrangements has been built is reviewed. It is shown that in the production of these mutations one individual atom-change must initiate a chain of reactions in two or more directions, so that two or more different but nearby breaks of the chromonema are induced, with subsequent fusion of ends derived from the different breaks. It follows from this mechanism that the atom whose change initiates a mutation does not necessarily lie within the gene in which the final change is produced, contrary to the premises of those who have calculated "sensitive volumes."

8. The question is again brought forward of whether gene mutations may be regarded as of essentially the same nature as minute rearrangements, though of a size too small to be visible cytologically, and it is shown that this is closely associated with the question of whether genes may be regarded as having definite boundaries delimiting them from one another. Several lines of evidence appearing to favor the idea of a certain similarity between minute rearrangements and gene mutations are presented. At the same time, several other lines of evidence herein cited give apparently conclusive reasons for inferring that the breaks occurring in both gross and minute rearrangements do not separate individual amino-acid units but take place between much larger structures. As yet, the attack on these problems remains in a very preliminary stage.

9. It is pointed out that previous work on the induction of mutations in *Drosophila* by chemical means has so far failed to give any marked rise in the mutation frequency. The most recently reported result—that of a high effectiveness of thymonucleic acid, added to the food of larvae, on the mutation process—could not be confirmed in experiments undertaken by us. However, these and other negative results are not to be taken as signifying that the chemical attack is destined to fail for, owing to the natural obstacles in the way of such work, it may hardly be said to have seriously begun.

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³This is not intended to be more than a fragmentary list of literature on induced mutations in *Drosophila* and related matters, citing some of the main works dealing with points given special attention in the above paper. For lists of literature that aim to be more nearly complete on this of literature on induced mutations in *Drosophila* and on re-subject, see Muller, 1928, 1930a and 1934a, Hanson, 1933, Oliver, 1934, and Timoféeff-Ressovsky, 1931, 1934a and 1937.

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DISCUSSION

FANO: While computing the "sensitive volume" for w , did you consider all white mutants obtained, or only those without any deficiency?

MULLER: The volume would not be radically altered, as about $\frac{2}{3}$ of whites found after irradiation of sperm are homozygous viable.

DEMEREK: What is your criterion for a terminal deficiency?

MULLER: It is hard to prove that a deficiency is terminal. I doubt that an individual case would prove the point, unless by the ultraviolet photog-

raphy or electron microscope methods, since the genes are vanishingly small in size.

DEMEREK: What is your opinion of Dr. Sutton's cases?

MULLER: I was referring to these. Actually the supposed "terminal deficiencies" reported by her and by previous workers have fallen into two classes: 1) those with bands lost only in the terminal region of the X, which appears to be of a heterochromatic nature and therefore highly subject to compound breakage, as well as being dispensable; and 2) those with a break further back in the X, in the euchromatin, with the terminal piece apparently exchanged with heterochromatin from elsewhere, as judged by the presence of a nucleolus organizer at the present end and by other evidence such as the apparent attachment of the piece that had been removed from the left end of the X to the stump of the short arm of the X or of the fourth chromosome.

KAUFMANN: Your suggestion that a terminal nucleolus results from the displacement of the nucleolus organizing region might be checked by a study of the prophase of mitosis in ganglion cells. Has this been done?

MULLER: No, I have only considered the published reports.

SUTTON: I have not looked at the ganglion cells. In the salivary cells, I find that the nucleolus organizer region at the base of the X is normal, and there is this nucleolus-like structure in addition at the end. In one case, N 264-113 translocation, nothing seems attached to the end.

MULLER: More analysis is needed in that case, for—despite some genetic data regarding the behavior of shaven, which is difficult to interpret—the cytological picture indicated that the left end of the X had become attached to the stump of the short arm of the fourth chromosome. If you could not prove this idea by individual cases you would have to find that the frequency of apparent terminal deficiencies was greater than that expected for non-terminal deficiencies in which the terminal section was invisibly small. I think that some quantitative evidence of this sort, adverse to the idea of terminal deficiencies, was reported in an abstract by Miss M. Bishop on work done in Texas several years ago.

FANO: Miss M. Bishop, working at Cold Spring Harbor, found a few terminal deletions with the break between *f* and *car*. If one assumes that the break distribution is random and that their frequency is proportional to the dosage, the frequency obtained by Miss Bishop can be compared with that of the *y* deficiencies found by Dr. Sutton, in which the break is to the right of *y* but not so far to the right as to be dominant lethal. The comparison shows that the two results are in good agreement. If one assumes further that the total frequency of single breaks is so large as to account for most of the dominant lethals, this frequency can be approximately determined by dominant lethals data, and

appears to be about 20 times larger than the frequency of terminal deficiencies. Thus approximately one out of every 20 single breaks would get "stabilized" so as to behave as a permanent "terminal deficiency."

Miss Bishop considered whether her "terminal deficiencies" might be long deletions in which all the region from *f* to *y* inclusive is deleted. It may be computed that the frequency of such deletions with one break between *y* and the tip is so small that it cannot account for the observations.

I did not understand the quantitative argument on the frequency of the "lost losses," but inferred that this meant that the frequency of the rod losses is deduced from the frequency of the ring losses.

MULLER: It was assumed that the frequency of breaks was the same in rod and ring chromosomes.

LURIA: In connection with the utility of the hit theory in supplying information about the size of submicroscopic biological entities, I should like to point out that encouraging results are obtained in the case of bacteriophages and viruses. Bacteriophage inactivation by radiations fulfills all requirements of the hit theory. Moreover, the sensitive volumes calculated from experimental results are nearly equal to the volumes of the phage particles themselves. That is true for tobacco mosaic virus too: both Lea and Gowen's data permit one to calculate a sensitive volume corresponding to a molecular weight of about 35 million, in fairly good agreement with Dr. Stanley's results. In the case of bacteriophages we also find complete independence of the inactivation from the wave length of X-rays, quite comparable with the result on production of X-chromosome lethals in *Drosophila*.

Differences in the way of calculating the sensitive volumes do not change the correspondence of order of magnitude of sensitive volumes and actual sizes.

It is interesting to see that Dr. Carlson's data, interpreted in the same way, should bring out the quite reasonable conclusion that a chromosome break in the grasshopper follows a hit in any of about 50 to 100 particles of the same size of a bacteriophage particle.

DELBRÜCK: I believe that the results quoted by Dr. Luria are indeed of importance for a discussion of radiation effects on chromosomes. Dr. Muller in his paper mentioned the many ambiguities which enter into the prediction, on *a priori* physical grounds, of the effects of radiation. These ambiguities arise partly from our lack of knowledge of the autonomous chemical reactions of the chromosomes, which may be upset by the radiation, and partly by our lack of knowledge of the primary chemical acts produced by the radiations. It is in the former respect that the experiments with viruses and phages can serve as a model experiment, where we may observe clearly separated that part of the radiation effects which leads to an interference with the process of reproduction. We learn from these experiments that the capacity for reproduction is

extremely sensitive, practically every ionization occurring within the self reproducing entity leading to its destruction. This type of radiation effect forms therefore a baseline of which we can be reasonably certain and on which other effects may be superimposed in the case of the chromosome.

GOWEN: The effects of neutrons versus those of X-rays should be considered.

MULLER: Dempster, in a work at the moment in press in the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, finds less ($.75\times$) effect of neutrons than of X-rays in producing gene mutations, but approximately the same effect (slightly greater, $1.25\times$) on gross rearrangements, and a greater effect ($1.5\times$) on "dominant lethals."

THE COMPARISON OF ULTRAVIOLET AND X-RAY EFFECTS ON MUTATION¹

L. J. STADLER

The chromosomal derangements induced by ultraviolet treatment of maize pollen are apparently qualitatively different in various ways from those induced by X-ray treatment under the same conditions (Stadler, 1941). This encourages the hope that the mutations also may be qualitatively different. It is now clear that many of the supposed mutations induced by X-ray treatment are due to chromosomal changes, and it is a matter of inference how large a proportion of the remainder may be analogous extra-genic alterations below the level of detectability with present technic. It remains to be seen whether this is true also of the ultraviolet mutations.

In the discussion which follows, the term "mutation" refers to variations behaving as if due to a change in a gene; in other words, mutations as experimentally identified. "Pseudo-mutations" are variations otherwise identifiable as mutations, which are demonstrably due to extra-genic alteration. "Mutations" are thus by definition alterations of unknown nature. The hypothetical transformation of a gene to an allelic form is referred to as "transmutation."

Since so many pseudo-mutations are found to simulate the effect of transmutation, one becomes suspicious of the inference of gene transformation in the mutations free from obvious chromosomal alterations. The demonstrated pseudo-mutations under X-ray treatment include losses of genes, additions of genes, and changes in the spatial relations of genes. Each of these phenomena may occur on a scale which would be cytologically undetectable.

This suggests the possibility that the X-ray mutations may be wholly or largely extra-genic alterations (Stadler, 1932). The results with maize (in which there are clear differences between X-ray and spontaneous mutations) do not indicate that this is true of mutation in general, but rather that the X-ray mutations are a special class. In *Drosophila* differences between X-ray and spontaneous mutations have not been obvious, and Goldschmidt (1940), assuming that the X-ray mutations are representative of the phenomenon of mutation in general, is inclined to dispense with the notion of intra-genic variation altogether.

If there are differences in kind between ultraviolet and X-ray mutations, these differences would not be so easily recognized as the differences between the chromosomal alterations induced by the two agents. Standard methods for determining the

effect of a treatment upon mutation tell little about the mutations induced except the frequency of their occurrence and the relative frequency of various phenotypic effects. The basic purpose of the radiation experiments is to affect the processes by which new genes arise, not merely those by which old genes are lost or masked. But we have at present no technic for distinguishing positively between mutations of the two classes. It is possible however to distinguish certain types of mutation which may differ in probability of relationship to chromosomal derangement, and it is possible to compare the frequency of these mutational types under the conditions of different treatments or of spontaneous mutation.

The purpose of this paper is to consider the possibilities, with maize, of developing criteria for the more critical comparison of the mutations induced by ultraviolet and X-ray treatment.

THE GENETIC NATURE OF X-RAY MUTATIONS IN MAIZE

The routine technic used in determining the mutational effects of radiations in maize has some advantage in the avoidance of extragenic alterations. It consists simply in the determination of the frequency of "visible" mutants in F_2 . A mutant can not appear in F_2 unless it has gone through the haploid gametophyte generation in both male and female transmission. Thus genic as well as chromosomal alterations with "haplo-lethal" effects are automatically eliminated. Even semilethal effects and relatively slight viability changes tend to be eliminated, since pollen tube growth is highly competitive. The mutations which appear in F_2 therefore are a highly selected sample.

These mutations are not associated with translocation. In the F_1 plants from treated pollen, tests for translocation and for mutation may be applied to the same plants. There is no correlation in the occurrence of mutations and translocation, and no case has been found of a mutation at a translocation point. Presumably if mutant effects occur at translocation points in maize the resulting mutations are eliminated in the gametophyte generation. A search for gametophytic effects associated with translocation was made by special methods, and none was found. We have at present no evidence of the occurrence in maize either of position effects or of deficiencies or mutations at translocation points.

The mutations however may be related to deficiency. The mutations as a group could be considered intra-genic only on the assumption that any deficiency would be lethal or at least distinctly deleterious to the gametophyte. This assumption is

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no longer tenable. It is true that typical deficiencies are haplo-lethal, in both the male and female gametophyte. But several minor deficiencies, long enough to be readily detectable at pachytene, are not wholly lethal. A rather long deficiency including the gene R' , which is partially haplo-viable, was described several years ago (Stadler, 1933). In this case the ovules are not wholly aborted, and on pollination by the normal a few heterozygous seeds are produced. These are reduced in size but are normally viable. They produce plants heterozygous for the deficiency, which are normal in development though slightly slower in growth than their non-deficient sibs. The deficient pollen is not aborted but is definitely subnormal in development. Some of the deficient pollen grains germinate. They never accomplish fertilization, and it is not possible therefore to determine whether the homozygous deficiency would be viable.

Several other haplo-viable deficiencies have since been found (Stadler, 1934; Creighton, 1934; unpublished evidence of several investigators). These represent various levels of viability, ranging to full female fertility with heterozygous seeds and plants wholly normal in development. The least defective cases have pollen also normal in appearance, though in almost all cases the deficient pollen fails to function in competition with the non-deficient. The only case reported with normally functioning pollen, so far as I know, is a very short deficiency apparently including the Yg_2 locus, reported by Creighton (1934).

There are also numerous cases of small pollen or slightly subnormal pollen in which no deficiency is cytologically detectable. In these also the small pollen is ordinarily non-functional, though in a few instances the small pollen factor is transmissible through pollen in much reduced frequency.

Among the recessive mutants which appear in F_2 from X-rayed pollen, there is a considerable number in which the segregation ratio is considerably less than 25 percent. In several of these, male and female transmission tests show that the low ratio is due to reduced transmission through the pollen, though the pollen in these cases is normal in appearance.

It is possible to assemble a continuous series of X-ray-induced variants ranging by hardly perceptible steps from the genetic behavior characteristic of the typical deficiency to that of the typical mutation. Those which result in segregation of a recessive phenotype in F_2 represent alterations which are transmissible through the male and female gametophyte. These are the alterations which are classed as mutations in the routine technic for determining mutation frequency at unmarked loci. They may consist merely of the selected survivors from the population of deficiencies of varying viability.

On the other hand, if new genes are produced by the action of the radiation, it is to be expected

that some of them will express their effects in the gametophyte. These might include variations completely haplo-lethal as well as sub-lethal—that is, they might include alterations inherited in the manner of the typical deficiencies mentioned above. In other words, a treatment producing deficiencies without transmutation might produce the entire series of viability variants mentioned; but a treatment producing transmutation without deficiency might do the same.

Among the alterations induced at a given locus by X-ray treatment, only a very small proportion survive the gametophyte generation and appear among the mutations in F_2 . Extensive data on this point are now available only for alterations at the A locus. Loss of the A effect may be identified in the seedling stage, and it is therefore possible to make the determination on large numbers. We have identified about two hundred of such seedlings. Those due to deficiency of A or inviable mutations should show corresponding defects in fertility and development; those due to wholly viable mutation should be normal plants of full fertility, except for the coincident occurrence of derangements induced at other loci. The errors from coincidence may be avoided to a considerable extent by the use of low doses, and in the case of plants which survive to flowering by outcrossing to recover the viable mutant.

Among the A losses so identified, the great majority were found to be distinctly defective plants which failed to reach the flowering stage. Among those which survived to flowering, the majority were visibly defective in development, and in all but a few the defective pollen for which they were segregating was of the extreme aborted type designated "empty" or "nearly empty." Those not visibly defective in plant development included cases with pollen of varying degrees of defectiveness, and included a few in which the pollen was subnormal but not aborted, and a few in which the pollen was indistinguishable from the normal. The last class, plants with no visible change except loss of the A effect, may be regarded tentatively as mutant a genes resulting from X-ray treatment. They constituted about 2 percent of the " A -losses" observed.

A large series of A -losses, grown in 1938, included two plants of this type. The "mutant a " in each case was recovered and crossed with A . The heterozygous plants thus produced showed in both cases full transmission through female germ cells but much reduced transmission through male germ cells. Thus this extensive trial failed to yield a single case of mutation to the typical recessive a .

We might expect that the mutant alleles of a given locus should vary in viability, and that in a series of A -losses like that described many of the cases with defective pollen, as well as those with normal pollen, might represent new mutant a alleles. While it is true that no defects of pollen development or function are associated with the standard alleles

of *A*, this might be due simply to the selective survival and establishment of the most viable mutants.

But in maize this expectation is contradicted by the results with spontaneous mutations, so far as the genes represented in the spontaneous mutation experiments are concerned. Eight dominant genes affecting endosperm characters were used, and spontaneous recessive mutations identified in extensive pollinations over a series of years (Stadler, 1930, 1933). Spontaneous mutations were found at all but one of these loci, and numerous instances were found at several of the loci tested. There was no association of partial sterility or of defective pollen development with spontaneous mutation at any of the loci. In the case of the mutations of *R'*, since the mutant is distinguishable from the bottom recessive at the locus, it was possible also to test transmission of the mutant through male germ cells in competition with the standard recessive allele. This was done for a large number of *R'* mutations, and there was no indication in any case of reduced transmission associated with the mutation.

These experiments with spontaneous mutation gave another result which is significant for the problem we are considering. The gene *R'* determines the occurrence of anthocyanin pigment in the endosperm and in the plant. In the presence of the recessive allele *r'* the pigment is absent in both endosperm and plant. There are intermediate alleles, including *R''*, with pigment present in the endosperm but not in the plant, and *r''*, with pigment present in the plant but not in the endosperm. It turns out that spontaneous mutations of *R'*, identified by loss of endosperm color, are regularly mutations to *r'*, not to *r''*. In a group of about 50 mutations of *R'* there were no exceptions to this rule. Mutations resulting in the loss of plant color without loss of endosperm color would not have been detected in these experiments. In subsequent experiments, in which mutation of *R'* was determined by seedling examination, it has been found that mutations eliminating the plant color effect also occur at comparable rates and that these are exclusively mutations to *R''*, not *r''*.

This provides for one locus an extremely sensitive test for discriminating between deficiency and mutation of the type which occurs spontaneously. The gene *R'* behaves in spontaneous mutation as two genes completely linked, or as a complex gene with two independently mutable parts. Whether it is one gene or two, deficiency incidental to chromosomal derangements would be expected ordinarily to remove it entirely and to lead to mutations or to pseudo-mutations of the *r'* phenotype.

DIFFERENCES IN THE CHROMOSOMAL EFFECTS OF ULTRAVIOLET AND X-RAY TREATMENT

The differences in the chromosomal derangements induced by ultraviolet treatment may be summarized as follows:

1) *Deficiencies*. Among the deficiencies identified phenotypically in endosperm tissue, the proportion of fractionals is much larger with ultraviolet treatment than with X-ray treatment. Unlike the X-ray fractionals the ultraviolet fractionals consist chiefly of seeds in which the distribution of deficient and non-deficient tissue is approximately equal.

The ultraviolet deficiencies identified in the *F*₁ plants are very much lower in frequency than those identified in the *F*₁ endosperms.

Among the ultraviolet deficiencies which have been studied cytologically, no case of intercalary deficiency has yet been found. Since in maize intercalary deficiencies may sometimes have the cytological appearance of terminal deficiencies this evidence is not conclusive. It indicates however that intercalary deficiencies are much less frequent with ultraviolet than with X-ray treatment, and this is supported also by the limited genetic evidence available.

2) *Translocations*. Simple interchanges are rare under ultraviolet, though very frequent under X-ray treatment. Such interchanges have previously been found occasionally in untreated material, and there is no evidence that their frequency following ultraviolet treatment is in excess of the spontaneous frequency.

It is clear however that ultraviolet treatment does effect the occurrence of translocation. Among the deficiencies found in progenies from ultraviolet treated pollen, there are certain plants with meiotic configurations showing translocation associated with deficiency. In these the association complex at diakinesis is a chain rather than a ring. In many instances the chain consists of three rather than four chromosomes; in others it may include four chromosomes, one of which is deficient. With heavy doses and with special precautions to avoid the elimination of defective plants, enough cases of deficiency-translocations are found to show clearly that they are a result of the treatment.

These contrasts suggest that there is a qualitative difference in the initial alterations produced by the two types of radiation, as might be expected from their physical characteristics. Much further study is needed to show the nature of this difference. From the effects already noted, one may hazard the guess that in the induction of the ultraviolet derangements there may be a considerable interval between the initial "break" or "hit" and the separation of the resulting chromosome fragments. This may account for several of the distinctive peculiarities of the ultraviolet effects. When two breaks occur in the same cell, separation may occur at one breakage point and be followed by loss of the acentric fragment, before separation at the other breakage point has made translocation possible. Free recombination of broken ends could then result only in a deficiency-translocation. When the two breaks are in the same chromosome this factor would tend to restrict the opportunities for the occurrence of

intercalary deficiencies and inversions. The extreme difference in frequency of deficiencies in endosperm and embryo is astonishing if the deficiencies are determined at the time of treatment, since the structure and exposure of the two sperms irradiated in a single pollen grain are so similar. But if fragmentation may be deferred for some time after fertilization it would not be surprising if its frequency were modified differentially, for the condition of early development in the embryo and in the endosperm are very different. The contrast in frequency of fractional deficiencies, if it must be accounted for on the basis of directly induced fragmentation, requires the assumption that the chromosomes are predivided and that the X-ray treatment usually affects both strands together while the ultraviolet usually does not. If realization of the ultraviolet breaks is determined by conditions after treatment, it is possible that the chromosome is undivided when treated and that the ultraviolet breaks are usually not realized in both daughter chromosomes.

THE RELATIVE FREQUENCY OF MUTATION UNDER ULTRAVIOLET AND X-RAY TREATMENT

It is impossible to make a direct comparison of the effect of the two radiations upon mutation frequency, since there is no basis for determining comparable doses without assuming a specific absorbing unit. It is necessary to make the comparison on the basis of some biological equivalent; for example, to determine the effect on mutation of doses equal in inducing deficiencies or translocations. But since the deficiencies and translocations produced by ultraviolet are of types different from those produced by X-ray treatment (or include various types in very different proportions), the doses equivalent on the basis of one chromosomal effect will be widely different from those equivalent on the basis of another. The doses used therefore must be chosen arbitrarily, and their equivalence may be judged only by the frequency of the various alterations in the progeny. In the experiments here reported, the ultraviolet doses used were close to the tolerance limit for the wave lengths represented. The X-ray doses were necessarily much below the tolerance limit, so as to

permit the survival of as many plants as possible and the production of well filled ears, which are essential for the determination of the mutation rates. The average X-ray dose and the ultraviolet doses were approximately equal in the frequency of plants segregating for defective pollen.

In populations so large as those required for the determination of mutation rates, it is not feasible to determine the frequency of chromosomal derangements by direct cytological examination of every plant. Some indication of the frequency of chromosomal derangements may be obtained from the frequency of the F_1 plants segregating for defective pollen and the type of the defective pollen grains. Segregation for defective pollen may result from translocation, from deficiency, or from gene mutations affecting pollen development. The evidence which already has been mentioned shows that deficiencies large enough for cytological detection result almost always in visibly defective pollen. There is additional evidence from previous experiments showing that translocation in maize regularly results in segregation for defective pollen; translocation with segregation so directed as to result in normal fertility does not occur with appreciable frequency.

One of the ultraviolet cultures and two of the X-ray cultures were included in a cytological study by Dr. Katherine O. DeBoer in which all plants segregating for defective pollen were examined at diakinesis for chromosome associations indicating translocation. The X-ray cultures included slightly less than 100 plants each, and were supplemented by others given the same treatment in order to increase the numbers compared in mutation frequency and pollen segregation. An additional ultraviolet treatment and a large control without cytological examination also were included in the mutation study. All plants were selfed and mutations determined for seed and seedling characters in all which yielded a well filled ear. All doubtful mutations were confirmed in F_3 . The data given are tentative in that a few of the confirmation tests have not been completed, and these doubtful mutations are given half-weight in calculating the mutation frequency. The data are shown in Table 1.

TABLE 1. RELATIVE FREQUENCY OF MUTATION AND CERTAIN OTHER ALTERATIONS FOLLOWING ULTRAVIOLET AND X-RAY TREATMENT OF THE POLLEN

Pollen Treatment	F_1 individuals which exhibited the alteration indicated.						
	Abnormal Meiotic Association		Segregation for Defective Pollen		Mutation		
	Inter-Change	Deficiency-Translocation	Observed Frequency	Adjusted Frequency*	Seed	Seedling	Total
	%	%	%	%	%	%	%
X-ray 250 r	4.1	2.1	12.8	15.4	0.6	0.3	0.9
X-ray 500 r	13.3	7.2	31.4	41.0	4.9	0.8	5.7
U V, λ 2967	0	2.2	19.9	22.8	10.8	6.2	17.0
U V, λ 3022			20.1	23.3	11.3	11.2	22.5
Control			0.9	0.9	0.7	0.3	1.0

* Assuming 2 factors for pollen segregation in plants with 75 percent or more of defective pollen.

In spite of the relatively low doses of X-ray treatment, translocations occurred with appreciable frequency, and even at 250 r were clearly more frequent than with ultraviolet treatment.

The relative frequencies of deficiency in the X-ray and ultraviolet cultures can not be determined, but the frequency of plants segregating for defective pollen gives some indication. It is possible also to draw some general inferences from the type of defective segregates. "Aborted" pollen is found both in translocations and in deficiencies; "subnormal" pollen seldom if ever in translocations but often in the shorter, intercalary deficiencies; "small" pollen (otherwise normal in development) rather seldom in plants with any cytologically visible deficiency. Both "subnormal" and "small" pollen segregations occur frequently in plants without cytologically visible deficiency.

The total frequency of plants with pollen segregations following ultraviolet treatment was considerably lower than that following an X-ray dose of 500 r. Among these plants the proportion in which the defective pollen was subnormal or small, rather than aborted, was much higher with ultraviolet than with X-ray treatment. If segregation for aborted pollen only is considered, the frequency of pollen segregation from ultraviolet treatment is about equal to that from the low X-ray dose of 250 r.

Since intercalary deficiencies are so rare with ultraviolet, it seems probable that the pollen segregations of the subnormal and small types induced by ultraviolet may be largely the result of mutations affecting pollen development rather than cytologically detectable deficiencies.

The mutation rate, as determined from segregating seed and seedling mutants in F_2 , is very much higher from ultraviolet than from X-rays at the doses compared. Assuming that the X-ray mutations are in some manner by-products of the X-ray derangements, the number of analogous mutations to be expected from the ultraviolet doses used is but a negligible fraction of those observed. This, of course, does not preclude the possibility of extra-genic mutations of other types induced by the ultraviolet treatment.

The mutation rate from ultraviolet is surprisingly high. Actually it is much higher even than the high rates indicated by the data in Table 1. Among the pollen grains treated with ultraviolet, because of the high absorption in passing through the pollen grain contents, only a small proportion receive a heavy dose at the site of the gametic nucleus. Assuming random orientation of the pollen grains, a large proportion have the nucleus at such depth below the treated surface that practically no ultraviolet energy reaches it. The measured rates therefore are the average from a mixture of variably treated and virtually untreated individuals. Calculations from the results of ultraviolet dosage experiments, with effects shown as endosperm deficiencies, indicate that the frequency of induced alterations

in the more favorably oriented pollen grains is several times as high as the mean frequency observed in the total sample treated (Stadler and Uber, in press).

Gametophyte Mutation. If segregations of subnormal and small pollen are in part the result of mutations of gametophytic effect, it may be suspected that there are also gametophytic mutations affecting the functional efficiency of the pollen but with no visible effect on pollen grain structure or development. In fact it would not be surprising *a priori* if such mutations were more numerous than the entire class of detectable sporophytic mutations. With respect to gametophyte viability the spectrum of mutant types to be expected ranges from that with no effect on gametophyte viability to that with complete inviability. In the routine technic the mutations at or near the one extreme are identified as mutations in F_2 , those at or near the other as factors causing pollen segregation and partial sterility. The middle range, mutants without visible effect on the pollen but with distinctly reduced pollen transmission, are overlooked except when they happen to be closely linked with a gene-marker used in the experiment.

The relative frequency of these gametophyte mutations under X-ray and ultraviolet treatment is of interest in connection with the problem of their origin. In the experiment just summarized, seed and seedling mutation are much more frequent under ultraviolet treatment, while alterations resulting in segregation for aborted pollen are more frequent under X-ray treatment. Are the gametophyte effects similar to the former or the latter in frequency?

A special comparison therefore was made in an experiment designed to identify gametophyte mutations. This may be done by the use of marked chromosomes with a heterozygous inversion to inhibit crossing over. McClintock's complex rearrangement of chromosome 9 (McClintock, 1939) was used, with the markers *I* and *wx* in the normal chromosome opposite. This permits the identification of gametophyte alterations within the rearrangement or nearby, by modification of the F_2 *I* and *Wx* ratios from 3:1 toward 2:2 and 4:0 respectively. In the case of derangements lethal to the female gametophyte, the ear is semi-sterile and the surviving seeds are all *i Wx*. Chromosome-9 alterations with slight effects on pollen size and development may be recognized with special precision, since the *Wx* and *wx* pollen grains are readily distinguishable with iodine staining. Those with no visible effect on the pollen are detected in F_2 only by the modification of the *I* and *Wx* ratio. A zygotic lethal, if it prevented development of the pericarp, as well as the embryo and endosperm, could produce a similar modification of the F_2 ratio, but could be distinguished in the following generation by comparative transmission in the selfs and outcrosses. In the present discussion these are tentatively classed with gametophyte alterations.

The frequency of gametophytic alterations thus determined may be compared with the frequency of chromosome 9 seed and seedling mutations detected in F_2 , since these are identified by linkage with I and wx in the F_2 ears. Finally all of the types of alteration mentioned, if they occur in the untreated normal chromosome 9 may be similarly recognized by opposite linkage with the same markers.

The alterations are induced in the normal treated chromosome ($I wx$). Any alteration not completely haplo-lethal may be recovered in F_2 , and may be crossed with a normal chromosome 9 stock with $i Wx$ for genetic location of the alteration and for critical cytological examination. The region is genetically the best marked region of the maize complement, and is also quite favorable cytologically.

The results as to pollen segregation and mutation at unmarked loci were similar to those of the experiment previously mentioned. The doses applied were different, particularly in that a higher X-ray dose was used. The relative frequencies of the chromosome 9 alterations were as shown in Table 2.

TABLE 2. RELATIVE FREQUENCY OF CERTAIN ALTERATIONS IN THE TREATED AND UNTREATED CHROMOSOMES 9 PAIRED IN 1114 F_1 INDIVIDUALS

Kind of Alteration	Alterations detected in the chromosomes subjected to the treatment indicated			Untreated Chromosomes
	Treated Chromosomes			
	UV $\lambda 2967$	UV $\lambda 2537$	X-ray 600 r	
Population	No. 486	No. 292	No. 336	No. 1114
<i>Gametophyte Alterations</i>				
Pollen aborted	3	0	5	0
Pollen subnormal	0	0	1	0
Pollen small	1	0	1	0
Pollen normal	11	4	2	0
<i>Recessive Mutations</i>				
Seed	4	2	0	0
Seedling	3	3	1	1

Although larger numbers would be desirable, the differential effect of the treatments upon pollen segregation and mutation is clearly evident in the chromosome 9 alterations. At the doses compared, the frequency of segregations for aborted pollen following X-ray treatment was several times as high as that following ultraviolet treatment, while the reverse was true of the frequency of seed and seedling mutations. These differences are confirmed with adequate numbers in the data for similar alterations at unmarked loci.

The gametophytic alterations without visible effect upon the pollen are distinctly more frequent in the ultraviolet than the X-ray series, their relative frequency being similar to that observed for the induced mutations rather than that for the induced pollen abortions.

Mutations of A. The extreme rarity of haplo-viable mutations of A under X-ray treatment already has been mentioned. In the large series examined, even the rare instances with visibly normal pollen were found to be gametophytically defective in some degree, as shown by their lowered male transmission.

This is not true of ultraviolet mutations of A . A much smaller number of A -losses induced by ultraviolet treatment has been examined, but among these have been found three cases of mutation apparently to the recessive a phenotype and one case of mutation to a new intermediate phenotype designated "light," A^{14} . These mutants are wholly normal in growth and in pollen development. All four have been tested in male transmission, and all are normal. In the remaining cases the defective pollen segregates were in all cases of the "aborted" type—none were "subnormal" or "small."

These results with X-ray and ultraviolet treatment are not strictly comparable, as they were obtained in separate experiments and in part with different stocks. In so far as they are comparable, they suggest a basic difference in the nature of the mutations induced by the two agents. X-ray treatment, tested on a very extensive scale, failed to induce mutation of A without some effect on gametophyte viability. This might be interpreted to mean that the potential mutant alleles of A are of varying levels of viability including few if any equal to the normal. If so, it would be expected that the ultraviolet mutants also would be of reduced viability. But mutants of normal viability do occur under ultraviolet treatment. Further, the partially viable mutants found with X-rays are absent in the ultraviolet series, though the significance of this difference will be uncertain until more extensive and comparable series of mutations from the two sources are available.

If the ultraviolet mutations result from transmutation and the X-ray mutations from haplo-viable deficiencies which include the A locus, the result is as might be expected. On this hypothesis the alleles of A would be regarded as normal in viability, and the reduced viability of the X-ray mutations would be ascribed to the loss of the genes which are included in the deficiency. The absence of haplo-viable deficiencies at the A locus in the ultraviolet series might be expected if ultraviolet deficiencies are usually terminal. A is at least 11 cross-over units from the end of the chromosome, and terminal deficiencies therefore would have to be fairly long to include it.

This of course would not imply that ultraviolet mutations and X-ray mutations in general differ in haplo-viability. Many known X-ray mutations are normal in viability, and the discussion just given of gametophyte mutation will furnish numerous examples of ultraviolet mutations of reduced viability. Comparisons of the mutations induced at specific loci are needed to determine whether significant via-

bility differences occur in the mutations induced by the two agents.

THE STUDY OF MUTATION AT SELECTED LOCI

A significant comparison of the viability effects associated with the induced mutations may be made at any locus. In addition, at certain loci, there are other advantages which may be of use in the determination of qualitative differences in the induced mutations.

The independent occurrence of mutations affecting plant color and seed color, in the spontaneous mutations of the gene R^+ , already has been mentioned. This serves to distinguish the spontaneous mutations of this gene from deficiencies. If ultraviolet mutations are analogous to the mutations which occur spontaneously, and if X-ray mutations are due chiefly to short deficiencies, the comparison of a series of mutations of the two types at this locus should show differences in phenotype as well as differences in viability.

The effects of the two newer alleles of A , found in South American maize (Emerson and Anderson, 1932) suggested that a similar advantage might obtain at the A locus. The allele A^b is similar in its effect on seed and plant color to the previously known dominant A , but in addition it produces a brown pericarp, dominant to the red pericarp of A . The allele a^p in its effect on seed and plant color appears to be intermediate between A and a , but it has the same dominant brown pericarp effect at A^b . This suggests that mutations may occur at the A locus, affecting seed and plant color without affecting pericarp color. If such behavior were the rule in spontaneous mutation, the gene A^b would have the same advantage as R^+ in the discrimination possible between mutation and deficiency.

A very important advantage of the A locus for the study of mutation resulted from the investigations of Rhoades (1938) with the gene Dt . Rhoades showed that this gene causes frequent mutation of a to A and other allelic forms, the mutations being unrelated to any visible chromosomal derangement. This indicates the possibility of an additional and crucial criterion, that of reversibility, for the distinction of mutation and deficiency at the A locus. It must be noted however that the standard a gene, which mutates under the influence of Dt , is derived from one or at most two original stocks, and there is no assurance that other a alleles arising by transmutation would respond in the same way. In fact, as Rhoades has shown, the a^p gene does not respond to Dt , and certain mutant forms of a which have arisen by mutation of standard a in the presence of Dt also are immune to its effect. Consequently, though reversibility of a mutant a under the influence of Dt is good evidence against deficiency, the failure of a mutant to be reverted by Dt is not good evidence against transmutation.

Because of the advantages mentioned, the A

locus provides the most favorable material for the critical comparison of the mutations arising under different conditions in maize.

Unfortunately the gene A was not included in the earlier experiments on spontaneous mutation, which have been mentioned. At the time these experiments were conducted, there was no reason for giving special attention to the A locus, and since it was not convenient to include a in the multiple recessive stocks this gene was omitted from the trials. Recently the frequency of spontaneous mutation of three of the alleles has been determined on a small scale, and extensive trials are now in progress.

Spontaneous Mutation at the A Locus. The phenotypic relations of the standard alleles are as represented below:

Allele	Aleurone Color (with R, C, A_1, i)	Plant Color (with B, Pl)	Pericarp color (with P)
A^b	Purple	Purple	Brown (dominant)
A	Purple	Purple	Red
a^p	Pale	Red-brown	Brown (dominant)
a	None	Brown	Brown (recessive)

Of these alleles only a is susceptible to mutation induced by Dt . The allele resulting from this mutation in most cases has the phenotypic effects of A , but there are several instances recently reported by Rhoades, in which the mutant represents a new dominant gene distinguishable from A but not identical with A^b or a^p . In addition Dt causes a high incidence of mutation of a to a form, phenotypically unchanged, which has lost the susceptibility to Dt -induced mutation.

The spontaneous mutations determined in this trial are limited to those that affect aleurone color. The studies on each allele tested were made on a single stock. The A stock was the one most extensively used in the radiation trials which have been mentioned, and all four of the A mutations induced by ultraviolet treatment occurred in this stock. The results of the spontaneous mutation trials are shown in Table 3.

TABLE 3. FREQUENCY OF SPONTANEOUS MUTATION OF CERTAIN A ALLELES

Allele	Mutation to Colorless Phenotype	Mutation to Pale Phenotype
A^b	0/55,765	19/36,661
A	0/53,226	0/40,952
a^p	0/13,478	—

It is clear that A^b is a relatively mutable gene, its mutation rate being of the same order as that of R^+ . The A gene and the a^p gene used in these experiments are much lower in mutation rate. They are not necessarily low in mutation as compared to other genes; the test is too small to have yielded mutants except in genes of relatively high muta-

bility. The absence of mutations of *A* in the populations used indicates that the ultraviolet mutations of this gene, which occurred in a smaller total population, were induced by the treatment.

The pale aleurone mutants obtained by mutation of *A^b* all give red-brown plant color resembling that due to *a^p*. The test of their effect on pericarp color, which requires an additional generation, has been completed only for four of the mutants. In all of these the pericarp color was brown, dominant to the red pericarp of *A*. This is the pericarp effect of the original *A^b* and of standard *a^p*.

In all cases the mutant plant was normal in development, the pollen was indistinguishable from the normal, and male transmission tests (which were made for almost every mutant) gave no indication of any gametophytic effect associated with the mutation.

Most of the mutants have been tested for reaction to *Dt*. The results were negative in all cases, as with standard *a^p*.

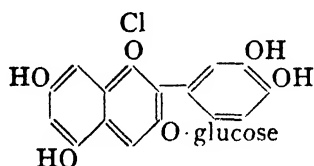
Thus the gene *A^b* has the same advantages as *R^r* for the critical comparison of induced and spontaneous mutations. It mutates spontaneously at a

alleles in terms of differences in the quality or quantity of some primary gene product.

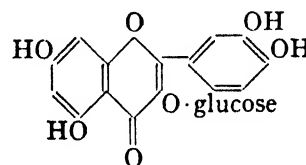
If specific loci are to be chosen for intensive study of mutation, a factor in the choice should be their adaptability to possible studies of gene action.

For various reasons the genes affecting anthocyanin pigmentation appear to be most favorable for this purpose. The studies made in recent years, chiefly by English workers (see review by Lawrence and Price, 1940) show that many genes affecting anthocyanin pigmentation in plants express their effect in a simple modification of the molecular structure of the pigment. The difficult task remains to determine the course of synthesis of the pigment, but when this has been accomplished it may be possible to investigate the role of specific genes in affecting the reactions involved.

Very little has been done in the chemical characterization of genetically determined pigments in maize. The only analyses reported for genetically known stocks are those of Sando and his coworkers (1922, 1935) on the husk pigment of Emerson's purple (*A B Pl*) and brown (*a B Pl*) types. The pigments identified are the following:



Chrysanthemins
A B Pl



Iso-quercitrins
a B Pl

high rate, its spontaneous mutations are wholly free from gametophytic effect, and they are phenotypically distinguishable from the results of deficiency at the same locus. In such trials, however, the *Dt*-reaction would not provide an additional criterion.

The pale mutants from *A^b* are not identical in phenotype with standard *a^p* or with one another. They vary in intensity of pigmentation, most of them being more deeply colored than standard *a^p*. They vary also in the degree of redness of the reddish-brown plant color. Their appearance suggests a mixture of red and brown pigments in different relative proportions in the different mutants.

Phenotypic Action of the *A* Alleles. Ultimately it may be possible to distinguish mutations by positive evidence of the presence of the mutant gene rather than merely by inference from negative evidence. The most promising approach to this is the analysis of gene action in terms of the effective agents produced under the control of the gene concerned. In the case of phenotypic effects expressible in terms of simple chemical differences, there is some ground for the hope that it may be possible ultimately to characterize the action of a series of

These pigments, associated with the difference between *A* and *a*, are the completely corresponding forms of anthocyanin and flavonol. If the difference due to *A* is analogous in the different pigments which are produced in other tissues and in the presence of other complementary genes, definite hypotheses may be formulated regarding the role of the gene in the synthesis of the pigments.

Effective study of gene action in the synthesis of anthocyanins may be possible by the use of the technic of excised root culture developed by Robbins and others. The isolated root fragment is cultured indefinitely on nutrient media consisting of inorganic salts, sugar, and certain "growth substances" of known constitution. McClary (1941) has recently described methods by which maize roots may be grown indefinitely on agar medium without the addition of any organic nutrient except sugar.

In the presence of the genes *A* or *A^b* and *R^r* or *r^r*, excised roots grown in this way synthesize anthocyanin abundantly. In certain genetic strains this pigment is chrysanthemins; in other strains other anthocyanins are produced. With *a* or *R^p* the roots are colorless. Presumably substances essential to

certain steps in the synthesis are produced only in the presence of certain alleles of *A* and of *R*. Since the root-culture technic permits complete control of the nutrients supplied, it makes possible direct experiments to determine what these substances may be.

The Identification of Sub-microscopic Deficiencies. Suppose that an extended series of mutations of *A^b* is obtained, including both spontaneous and induced mutations. Those which show visible chromosomal loss may be identified as deficiencies; those which show mutation to the pale type with no loss of the dominant pericarp effect as alterations other than deficiencies. Between these extremes are the cases of apparent mutation to the colorless *a* type, with loss of the dominant pericarp effect and perhaps with associated viability defects, but without cytologically visible deficiency in the chromosome. We may infer on the basis of the considerations which have been mentioned that these are in the main due to deficiencies too short for cytological detection. Is there any way in which this inference may be checked?

McClintock (1938, 1941) has shown that ring chromosomes spontaneously undergo alterations during somatic development, resulting in deficiencies and duplications of the genes present in the ring. When the segment present in the ring is not present in any other chromosome of the plant, the deficiencies resulting from non-lethal alteration of the ring result in sectors of homozygous deficient tissue. The characteristics of the homozygous deficient tissue, in sectors from different alterations, indicate the presence in the ring-segment of separable portions of specific phenotypic effect, and the combinations in which these characters occur indicate the order of these portions. The group-losses of the separable portions of the ring constitute genetic evidence of deficiency, which may be a more sensitive detector than cytological examination. It is not yet clear how large a part of the ring is occupied by the portions lost in the viable alterations.

These results suggest some possibilities for further study of the inviable mutants suspected of sub-microscopic deficiency, which could be made by the use of a ring of similar behavior but including the *A* locus. In those alterations which can not be made homozygous because of non-functional pollen or zygotic lethality, genetic evidence of deficiency is not obtainable. By the use of a ring including the *A* region, the gametophytic or zygotic inviability could be suppressed, and ring-bearing plants could be produced with the mutant present in both rod chromosomes. The somatic losses and alterations of the ring would then produce sectors "uncovering" the homozygous mutant, and these sectors would be homozygously deficient for any loci deficient in the mutation.

A ring chromosome including the *A* region has recently been found and appears to be well suited to this purpose. The sectorial distribution of *A*

is evident in aleurone as well as plant tissue.

Fortunately the allele present in the ring is *A^b*. This should make possible the extraction of mutants of *A^b* resulting from chromosome breakage, for comparison with those from induced and spontaneous mutation.

The Allelic Series at the R Locus. Four alleles of *R* have been mentioned, *R^r*, *R^o*, *r^r*, and *r^o*. Several others are known. In effect upon aleurone color, in addition to the full-colored and colorless types there are several partly colored types (*R^{nj}*, *Rst*, *R^{mb}*) and a colorless type of partial dominance (*r^w*). In effect upon plant color there is an additional colored-plant type, *r^{ch}*, which in the presence of *Pl* extends the purple pigment to the pericarp. Anderson (unpublished) has recently found a similar type with colored aleurone (*R^{ch}*).

The colored plant types designated *R^r* and *r^r* (Emerson, 1921) include various subtypes differing in distribution of the color in anthers and silks, and these differences have been found to depend on distinct *R* alleles, now designated *R^{ro}*, *R^{oo}*, *r^{rr}*, *r^{or}*, and *r^{oo}* (summary and references in Emerson, Beadle, and Fraser, 1935).

Among stocks with the colored-plant alleles, anthocyanin occurs in various organs other than the anthers and silks. When its distribution is plotted in detail a great number of distinguishable types is found, both among colored aleurone and colorless aleurone strains. A collection of colored aleurone strains cultivated by various tribes of American Indians, obtained through the kindness of J. H. Kempton, included many of these variations.

Five of the Indian strains, all classifiable as *R^r* but individually distinguishable by peculiarities of color distribution and intensity, were crossed to *r^{ch}*. The plant color distribution of *r^{ch}* is broader than that of any other allele at hand, while its aleurone is colorless. If the peculiarities of distribution characteristic of the *R^r* strains were due to distinctive alleles of *R*, the recovered *RR* individuals would show the same peculiarities; if they were due to modifying factors at other loci, the recovered *RR* individuals would show varying color distribution. The results show that the five *R^r* alleles are individually distinguishable, each determining the distribution of anthocyanin pigmentation in the plant in a distinctive way. It is probable therefore that an extremely large number of phenotypically distinguishable alleles or *R^r* and *r^r* exist among the established races of maize.

Similarly, in the effect upon aleurone color, there are differences between *R* alleles in the intensity of coloration as well as the differences in aleurone color distribution previously mentioned. Differences in intensity of aleurone color in the different *RR* races are of course subject to the effects of modifying genes, but there are cases in which crosses of the type *R^r R^o × r^o* permit almost perfect separation of the *R^r r^o* from the *R^o r^o* seeds on the basis of aleurone color alone. This may be done also with

certain R' stocks in which differences in plant color distribution make it possible to check the accuracy of the aleurone color separation.

Different R alleles also differ widely in their frequency of mutation to r (colorless aleurone). Mutation to a form with less intense aleurone color would not have been detected in the previous experiments unless the reduction in intensity were so extreme as to yield a nearly colorless allele. A few mutants to very faintly colored types were found.

In the spontaneous mutation of R' alleles, the mutations affecting aleurone color are to an r' allele corresponding in details of distribution to the R' allele mutating. The mutations affecting plant color which have been mentioned are those to an R'' allele—that is, those in which all plant color effect has been eliminated. The seedling color examinations in which these mutations were identified do not determine to what extent, if any, mutations occurred from the original R' allele to modified R' types. Detailed examination of plants grown to maturity will be required for adequate investigation of the mutations of this class. Fortunately the mutation rates at the R locus, at any rate for the changes of the types previously studied, are high enough to make the use of mature plant characters feasible.

Thus the R locus, like the A locus, has certain unusual advantages for the study of mutation and for the detection of pseudo-mutation. In certain respects the advantages of the two loci are complementary. An obvious advantage of the R locus is the great wealth of allelic forms available, with evidence of independently mutating units involved in certain series of mutant alleles. A further advantage is the probability that X-ray mutations at this locus will not be so regularly inviable as the X-ray mutants of A_1 since even the long deficiency 10-1, previously described, is partially haplo-viable.

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DISCUSSION

GATES: Your analysis is masterly, but I feel that a slight alteration in terminology is desirable; from the evolutionary point of view, it seems necessary to regard all germinal changes as mutations. Such things as deletions and duplications would then be one class of mutations; they are not pseudomutations. The term "gene mutation" would then be retained for this class of mutation.

STADLER: I see no objection to the use of the term mutation in the broader sense. For convenience in discussion I used the term in the narrow sense with the definitions given. I wish a substitute for the term "gene mutation" were available.

GATES: What is the matter with "gene mutation"?

STADLER: It seems to me absurd to call these changes gene mutations when the chief objective of the analysis is to find whether they involve a change in the gene or not.

SCHULTZ: Data concerning the rates of direct and reverse mutations at a number of loci, and particularly at the white locus, had been accumulated for X-ray induced mutations in *Drosophila* by a number of workers. In these cases it appears that the rate at which any of the mutant types appears in an X-ray experiment, as a mutation from one of the other alleles, is inversely related to the frequency with which it gives mutants to the other alleles when it is itself irradiated. Are there any suggestions of similar relations in the maize cases?

STADLER: No.

DELBRÜCK: Can the difference of mutation frequency between endosperm and embryo be ascribed to healing as in the case of McClintock?

STADLER: There is a distinct difference between endosperm and embryo in the sequelae of mechanical breaks and of ultraviolet breaks. We have not yet been able to find a common explanation for these two phenomena.

NEBEL: In the X-ray results, is the ratio of embryo to endosperm deficiency 1:1?

STADLER: No, but it is of that order, while with ultraviolet the frequency of embryo deficiencies is extremely low.

NEBEL: Is there any significant difference between ultraviolet and X-ray dosage curves if translocation frequency is plotted against dosage?

STADLER: The dosage curve for translocations cannot be determined because translocations are so rare. However, a dosage curve for deficiencies can be determined with considerable precision as far as numbers are concerned. But the dosage effect with ultraviolet is so greatly distorted by the

factor of unequal exposure in the different pollen grains treated that the comparison with X-rays would not have much meaning. The different pollen grains lie in a single layer during irradiation but the gametic nuclei are eccentrically located within the pollen grain and with casual orientation the more favorably oriented pollen grains receive a very heavy dose while the less favorably oriented ones are practically untreated. The earlier increments of dose produce their effects in the more favorably oriented grains while the later increments of dose can produce effects only in what is left over. There is consequently a marked flattening of the dosage curve which is more pronounced at some wave lengths than at others. This factor marks the effect of others which might be sought in the comparison of the dosage relations with ultraviolet and X-rays.

MULLER: Are the translocated chromosomes also deficient?

STADLER: Something is deficient in the translocation complex, but no one has yet succeeded in analyzing exactly what occurs in these translocations. Singleton and Clark have analyzed one case.

SINGLETON: Clark made most of the analysis, which concerned one plant where a three-armed configuration was involved, chromosomes 1 and 10 being translocated but we are not sure exactly what was missing.

MULLER: Would the assumption that a telomere had been formed on one broken end, leaving the other broken end free to unite explain such a case?

SINGLETON: Two other deficiencies, terminal and rather long, gave no translocations.

STADLER: Since none of the deficiency-translocations has been thoroughly analyzed, we can discuss them only in terms of what we think might be there. The observations of diakinesis and metaphase enable us to say only, 1) that translocation has occurred, in other words, that fragments from two different chromosomes have reattached; and 2) that this translocation is not reciprocal since the diakinesis configuration does not include two complete translocated chromosomes. In terms of the hypothetical mechanism which I mentioned we might suppose that when potential breaks are pro-

duced in two chromosomes, separation of the fragment is delayed to a varying extent and often the acentric fragment resulting from one break may separate and be lost before realization of the other break has occurred. The centric fragment from the first break may unite with either the centric or the acentric fragment from the second break. Either will produce a deficiency-translocation, the first yielding a diakinesis association of three chromosomes, the second an association of four chromosomes one of which is deficient. Such deficiency-translocations are produced also by X-ray treatment but the significant point is that with X-rays they are much less frequent than simple interchanges; with ultraviolet they are much more frequent. Simple interchanges are hardly increased above the spontaneous frequency by ultraviolet treatment.

PLOUGH: What is the relation of temperature and age to the different types of rearrangement?

STADLER: There are no data because corn pollen is very susceptible to both temperature and age, and one must give it the best possible conditions to get any results at all.

COLE: Are some differences between ultraviolet and X-ray explicable as differences in the primary event? Intermediate effects might possibly be obtained with short wave length ultraviolet radiation.

STADLER: Maize is poor material for biophysical analysis. Genetically known material is necessary, but another object is needed which would be suitable for genetical, chemical and physical studies.

FANO: One might say that ultraviolet-induced changes are more similar to spontaneous than to X-ray induced changes.

COLE: These lower wavelength ranges involve different electronic states and may even ionize the outer electrons of the molecules under consideration. It is conceivable that ionization of this kind may produce different genetic effects than excitation and possible ionization of substrates caused by the long r radiations between 2200 Å and 3000 Å. There are however many technical difficulties to be overcome in handling radiations below 2200 Å. Chief among these is, as you have indicated in your talk, the relatively poor transmission of the pollen grains.

WAVELENGTH DEPENDENCE OF MUTATION PRODUCTION IN THE ULTRAVIOLET WITH SPECIAL EMPHASIS ON FUNGI

ALEXANDER HOLLAENDER AND C. W. EMMONS

INTRODUCTION

The study of the biological effects of ultraviolet radiation on microorganisms has concerned itself for many years mostly with toxic effects. Such a method of attack was indicated by the theory that radiation produces its action by a simple physical mechanism and that this mechanism was responsible for the killing. It was thought to be an all-or-none effect. No sublethal effects were either looked for or found (Wyckoff, 1932).

Many chemical compounds particularly those which possess conjugated double bonds absorb ultraviolet radiation in distinct bands. In general, different structures are responsible for absorption in different regions of the spectrum, as for instance, the carbonyl linkage around 2800 Å, the conjugated carbonyl around 2400 Å, conjugated double bonds 2300-2900 Å. Ring structures with conjugated double bonds usually absorb in the region 2600-2800 Å. For instance, the benzene molecule has a set of well defined bands in this region. The specific absorption caused by these structures can be modified by substituted groups attached to the molecule (Brode, 1939). Ultraviolet radiation which is absorbed at these wavelengths can either be used for the breaking of the bonds responsible for the absorption or the energy could be used in producing heat, fluorescence or be transferred and produce its action in other parts of the molecule. The field of photochemistry has developed around the absorption and utilization of radiant energy.

It should be possible by means of selected wavelengths to affect different parts of certain molecules. It should also be possible in living materials to affect different activities of the cell by treating it with certain selected wavelengths. Chemical units which control certain functions of the cell can be changed differentially by different wavelengths. Of course, the final reaction to continued irradiation of the living material must be always the same, that is, death of the organism. Two physical conditions are important for the production of sublethal effects; the radiation if possible should be given in selected wavelengths and the energy values should be carefully adjusted to make certain that not all functions of the cell are stopped.

Microorganisms have many advantages for the study of the effects of ultraviolet radiation. They are small, so that we have no extensive penetration problem. They can be handled in large numbers and so provide good material for statistical tests. Further, they are of great importance from a medical and agricultural point of view. However, our knowl-

edge of their genetical makeup is rather meager; although more information has become available during the last few years in regard to certain fungi and yeasts. Our knowledge of the genetics of bacteria is extremely limited.

EFFECTS ON FUNGUS SPORES

We will first describe results obtained in our studies on the irradiation of fungi and then will compare these with studies on other plants, and finally with studies on *Drosophila*. Most of the genetical work done in our laboratory on microorganisms was conducted with the spores of a typical dermatophyte, *Trichophyton mentagrophytes*, isolated from dermatophytosis (ringworm of the arm) (Hollaender and Emmons, 1939; Emmons and Hollaender, 1939). The unicellular spores of this fungus appear to be uninucleate. While it has long been known that exposure to ultraviolet radiation, X-rays, heat, or chemicals may induce mutations in fungi, it is also recognized that mutations may occur independently of any recognized influences of these types. The apparently spontaneous production of mutations can be readily demonstrated by subculturing from spots or sectors of atypical growth sometimes found in old cultures. In order to avoid any possible confusion with spontaneous mutations in our present studies we used only young cultures in which, as determined by several thousand test subcultures, mutations had not yet appeared.

EXPERIMENTAL TECHNIQUE

We have developed for the irradiation of bacteria, yeasts, and fungi, a method which insured that each organism receives on the average an equivalent amount of monochromatic radiation (Hollaender and Claus, 1936). Radiation from a water cooled, high pressure quartz capillary mercury vapor lamp using one KV, was concentrated on the entrance slit of a large crystalline quartz monochromator. The emerging monochromatic beam was concentrated on the face of a vacuum thermopile connected with a high sensitivity galvanometer and standardized. The spores were suspended in a physiological salt solution non-absorbent for the wavelengths used in this investigation, and stirred thoroughly during the process of irradiation. Thermopile and exposure cell were kept in a constant temperature water bath. The density of the spore suspension insured that at most wavelengths all radiation, direct and scattered, was absorbed.

Our technique of irradiating liquid suspensions

was critically tested by experimental methods. These tests showed that when the exposure cell, which had a depth of two cm. and a capacity of eight cu. cm., contained a concentration of about 70 million spores per cu. cm., and when this spore suspension was rapidly and constantly stirred, the incident energy divided by the number of spores gave the average energy each spore received. The method therefore can be used for obtaining fairly accurate statistical data.

In practice a control was set up for each experiment and for each wavelength in the experiment by withdrawing from the free arm of the exposure cell with a sterile pipette 1/10 cc. of the spore suspension, diluting it in physiological salt solution, and plating out the sample on cornmeal agar. Decrease in viability as radiation continued was measured by withdrawing and plating out similar 1/10 cc. samples at appropriate intervals. The plates were incubated at 30° C for five or six days when the colonies which had developed were counted to determine the survival ratio. The mutation rate was determined by a random sampling method in which all colonies in a plate or in a sector of a plate were individually transferred to agar slants. Subculture on the agar slants was necessary in order to obtain an accurate count of the number of mutants among the surviving spores. Many of the mutants grow more slowly than the original type and if left to develop on the original poured plate will be quickly overgrown and hidden by more rapidly growing neighboring colonies. The conclusions presented are based on a study of some 50,000 colonies so analyzed. Large numbers of colonies must be studied

in order to obtain statistically significant data. It is of prime importance that a pure strain of the organism be used. To insure this, we used a strain which had been propagated from a single spore, and checked the genetic purity of many of the mutants by similarly establishing "single-spored" lines.

PHYSIOLOGICAL EFFECTS

The first apparent sublethal effect was a delay in germination of irradiated spores and for a variable period after germination the growth rate was retarded. These effects were measured by comparing the young colonies developing from irradiated spores with those arising from control spores. Colonies which were greatly retarded were picked and subcultured to determine whether this newly acquired characteristic was permanent and whether other correlated changes could be found. This type of retardation was found to be a temporary effect, subcultures growing at a normal rate, and no mutations were associated with it. These temporary changes are of no immediate interest in the present discussion of genetical effects.

MUTATION PRODUCTION

Before discussing the mutations induced by ultra-violet radiation it might be well to point out again that spontaneous mutation in this fungus is not unusual as a culture ages. We avoided any interference from this phenomenon, however, by using only young cultures in which mutations did not appear. Among 5,000 non-irradiated spores which we tested by culture in connection with the controls in each of our experiments, no mutants appeared.

TABLE 1. EXPERIMENT F-40—JULY 5, 1939
Trichophyton mentagrophytes, STRAIN No. 607—TEN-DAY CULTURE

Run	Number of spores per plate Average of three	Survival ratio percent	Ergs/spore absorbed	Number of single colony isolates	Number of mutations	Percent mutations
1	107.3	91	1.19×10^{-4}	80	0	0
2	91	77	2.88	80	0	0
3	75.7	64	4.83	80	0	0
4	77	65	6.80	80	0	0
5	63	53.4	9.29	80	1	1.25
6	82	69	11.81	80	0	0
7	65	55	15.39	80	6	7.5
8	65	55	18.48	80	1	2.4
9	52.3	30.5	22.14	80	5	6.3
10	31	19.5	25.85	80	7	10
11	17.3	11.9	29.60	80	11	13.8
12	9.2	7.8	33.94	80	11	13.8
13	5.5	4.65	38.34	80	20	25
14	2.9	2.46	42.80	80	17	21.3
15	1.4	1.19	47.89	80	11	13
16	.537	.456	53.6	80	8	10
17	.18	.153	59.98	80	10	12.6
18	.117	.1	67.60	80	4	5
19	.096	.081	76.55	80	9	11.2
20	.0185	.0157	88.75	80	12	15
Control 1	121	100	0	80	0	0
Control 2	115	100	0	40	0	0

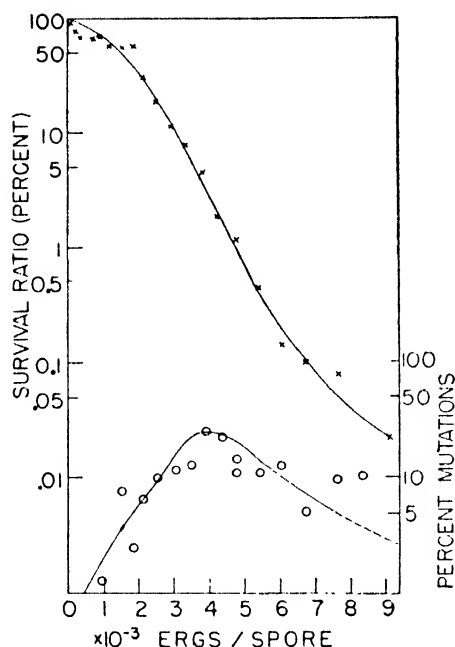


FIG. 1. Top curve: Change of survival ratio of fungous spores with increasing energy using 2650 Å radiation. Lower curve: Variation of percent mutations of surviving spores. Both curves have been obtained from the same material (see Emmons and Hollaender, 1940).

The induced mutations isolated in this study are characterized by a difference in kind or in degree of pigmentation, usually a decrease, in growth rate, or a difference in the amount of aerial hyphae. Certain types of mutants were repeatedly isolated, probably indicating a specific injury to some especially vulnerable part or function of the cell. The hundreds of mutants isolated could not be sharply classified among a few types because of the great variety of changes encountered. No positive correlation was observed between type of mutant induced and the wavelength to which the spores were exposed. There was, however, a clear relationship between the mutation rate and energy. This relationship is shown in Table 1 and Figure 1. It appears from this illustration as if very low energies do not produce any mutations. However, this is difficult to check. Carefully conducted tests on the effects of low energy values showed that the experimental error is so large that one cannot be certain that 1/10 percent of mutations are not present. Increasing energy will produce an increasing number of mutations up to a certain level. In this part of the curve we have an almost straight line relationship. The level of highest percentage of mutation is not always too certain. It apparently depends on a number of factors, the control of which we have not yet learned. The highest percent mutation observed at these maxima was 42 percent. We can count on obtaining 10 to 20 percent with greatest regularity. Still further increase of energy beyond this maximum will give a

smaller number of mutations. This decrease in mutation rate of surviving spores is rather surprising since it has not been reported for any other organism. The mutation rate usually will not return to zero but will fluctuate at a lower percentage level for considerable amounts of energy. An interpretation of this decrease of mutation rate is very difficult. One would think at first that we have not irradiated our spores uniformly and that the decreasing rate is produced by spores which have not been properly irradiated. Special tests conducted to check on this point, however, showed that the majority of the spores received an equivalent amount of energy because in our study of secondary effects (non-genetic) we have not found spores which reacted differently from the rest.

APPARENT INCREASE OF MUTATION RATE BY TREATMENT AFTER IRRADIATION

We have observed (Emmons and Hollaender, 1940) that when irradiated spores were incubated in solutions of phenol, iodine, or different salts after irradiation instead of being plated out immediately, there was a marked tendency to recover (Emmons and Hollaender, 1939). Some spores which had received an amount of radiation which should have been lethal (as determined by plating out samples immediately after irradiation) recovered when held for varying periods of time in appropriate solutions before plating out. Typical results are given in Figure 2. The recovery is quantitative and responds to different concentrations of chemicals. If we include the recovered spores in a typical mutation curve, we get the following set of curves, as shown in Figure 3. This diagram is an idealized composite

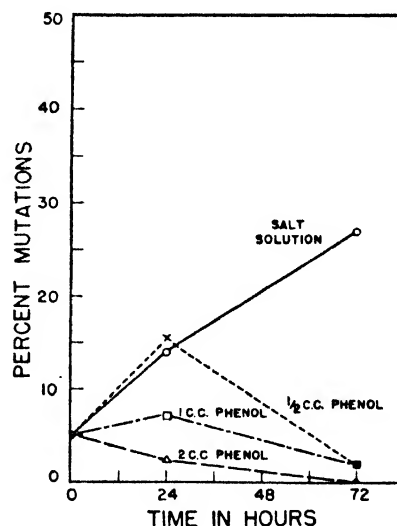


FIG. 2. Effect of incubation in certain solutions on mutation rate. "Salt solution" refers to physiological salt solution. The phenol solutions used were .05, .1, and .2 percent solutions respectively. For details see Emmons and Hollaender (forthcoming paper).

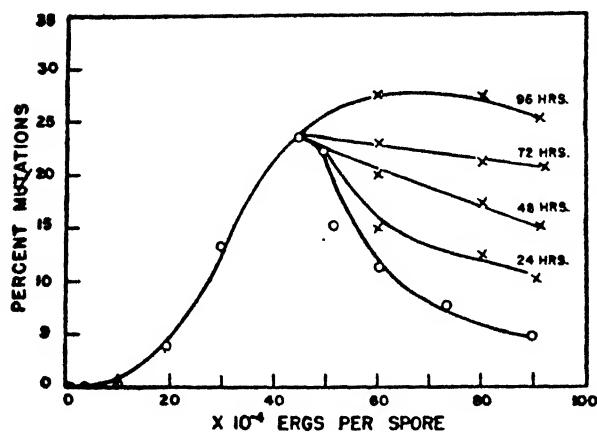


FIG. 3. Modification of mutation curve (taken from fig. 1) by incubation of spores in salt solution after irradiation for certain time intervals.

of a number of experiments. There are several explanations possible for this recovery process. 1) Treatment of the spores after irradiation may help to extend or complete the process of change initiated in the nucleus. 2) Spores which have received considerable amount of radiation often have a tendency, after incubation in liquid suspensions, to recover from the irradiation effect. It is possible that mutated spores will recover more readily than spores which have received extra nuclear injuries.

We want to refer here to a forthcoming publication by Emmons and Hollaender which discusses the process of recovery from irradiation effects.

WAVELENGTH DEPENDENCE OF MUTATION PRODUCTION

We have tested intensively the effectiveness of eight wavelengths between 2180 and 2967 Å in their ability to produce mutations (Hollaender, 1939). We used for these calculations only the straight part of the mutation curve as shown in Figure 1. Typical tables are given below. A plot of some of these data for 2, 4, and 6 percent mutation is given in Figure 4. To compare the wavelength dependence of mutation production with the fungicidal action of ultraviolet radiation, we are also giving the following figure (fig. 5), which gives fungicidal curves for 20, 40, and 60 percent killing.

There are several interesting features in these curves. First, 2650 Å appears to be the wavelength most effective in producing mutations as well as toxic action. The minimum at 2480 Å is the same for both actions. There is a slight maximum of effectiveness at wavelength 2280 Å, then with shorter wavelengths the effectiveness of radiation decreases. The 2650 Å maximum coincides with the high absorption coefficient of nucleic acids near this wavelength. This does not necessarily mean that nucleic acid is the only cell component responsible for this maximum. Proteins and certain enzymes

which are present in only very low concentrations could contribute very well to the maximum at this wavelength. The second maximum at 2280 Å is possibly caused by the absorption of these wavelengths by nuclear proteins. These compounds have almost continuous absorption below 2400 Å. The decrease of effectiveness of wavelengths below 2280 Å is probably caused by the protective action of the cell wall as well as the protective action of cytoplasmic material surrounding the chromatin. The protective action of cell wall is probably considerable at 2180 Å, since it has been reported (Schaede, 1939) that many fungi have cell walls made of chitin which as we have described in another place, has pronounced continuous absorption below 2200 Å (Durand, Hollaender and Houlihan, 1941). The resemblance between the mutation curve and fungicidal curves is close over most of the wavelengths, with the exception of two wavelengths tested. At 2180 Å the efficiency of the mutation action is greater than the fungicidal action. This is still more pronounced at 2967 Å.

Extensive tests were conducted to check whether the radiation longer than 3000 Å produces mutations. At wavelengths 3400 to 4400 Å only toxic

TABLE 2. MUTATION PRODUCTION

Wavelength	Energy necessary for production of mutation per spore		
	2 percent	4 percent	6 percent
2180 Å	4.8×10^{-3}	7×10^{-3}	10×10^{-3}
2280	2.2	4.2	6.0
2380	2.4	3.8	4.8
2480	3.6	5.4	6.8
2537	2	4	5.4
2650	0.8	1.6	2.2
2805	3	4	4.8
2967	14	20	25

Experiment F-11			
2180 Å			
2280	2.2×10^{-3}	5×10^{-3}	7.2×10^{-3}
2380	2.3	4.2	6
2480	4.3	7.8	10
2537	2.5	3.5	4.5
2650	1	3.0	4
2805	3	4.5	5.8
2967	10	17	20

action was observed. The energy necessary to kill at these wavelengths is many times the energy needed to kill at wavelengths below 3000 Å. The mechanism of killing at the long wavelengths is different from the mechanism of killing at 2650 Å (as found with bacteria) (Hollaender, 1940 and in manuscript). No mutations were observed with radiation longer than 3400 Å. The wavelength region around 3130 Å is still under investigation.

It has often been mentioned that the toxic action

and mutation production of ultraviolet radiation probably are the same, in other words, that the killing of microorganisms is a genetical effect. This is not necessarily correct because the relative efficiency of 2180 Å and 2950 Å for killing and for mutation production is not the same, as for instance at 2650 Å. Further, 3650 Å will produce toxic action without producing mutations.

It will seem more reasonable that toxic action and mutation production have a maximum of sensitivity at the same wavelength, that is, 2650 Å, and are not necessarily identical effects if we visualize that nucleic acid is not only a major constituent of the genetically "active" regions (euchromatic), but also of the genetically inactive (heterochromatic) part of the chromosome and regions of the cytoplasm immediately surrounding the nucleus (Caspersson, 1936; Schultz and Caspersson, 1939). It is very well

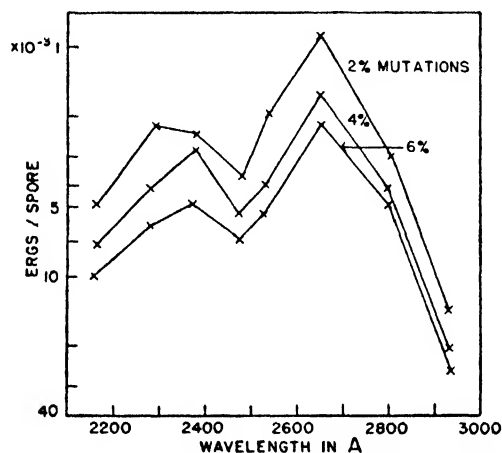


FIG. 4. Reciprocal of relative energy at eight wavelengths for 2, 4, and 6 percent mutations.

possible that when we inhibit cell division by means of ultraviolet radiation, the nucleic acid in the cytoplasm and heterochromatic region is mostly affected, whereas in the case of mutation production by monochromatic ultraviolet radiation, the euchromatic region in the chromosome responds to radiation. It is also reasonable to expect that in certain organisms in which nucleic acid in the cytoplasm protects the chromatin material, it would be difficult to produce mutations with ultraviolet radiation without inhibiting cell divisions.

ULTRAVIOLET PRODUCED MUTATIONS IN OTHER ORGANISMS

Noethling and Stubbe reported in 1934 the production of gene mutations after irradiation of the pollen of *Antirrhenum majus* with four wavelengths in the ultraviolet. Wavelength 2967 Å was the most effective in producing mutations. Little account was taken of the differential absorption of the different parts of the pollen grain; also the number of tests conducted was limited.

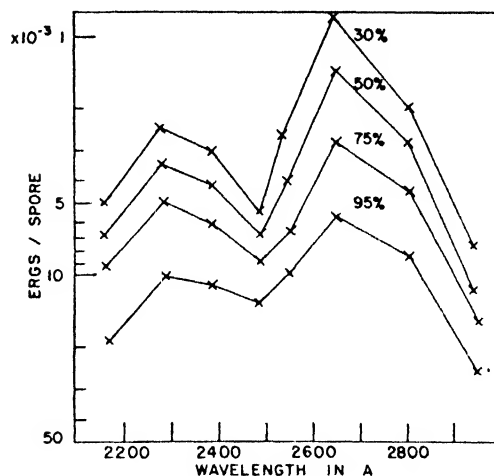


FIG. 5. Reciprocal of relative energy at eight wavelengths for fungicidal action for 30, 50, 75, and 95 percent survival ratios.

Knapp, Reuss, Risse, and Schreiber (1939) irradiated the sperm of *Sphaerocarpus Donnellii* with six wavelengths between 2537 and 3130 Å. They determined the toxic action and mutation production by tetrad analysis of sporangia in F_1 individuals. These data are reproduced in Table 3. The wavelength most efficient for both toxic action and mutation production was 2650 Å. Although the data given by these authors are not abundant, they are compared with our findings. Absorption spectra of sodium thymonucleate, the mutation spectra of *Trichophyton mentagrophytes* spores, and *Sphaerocarpus Donnellii* spores are given in Figure 6. All

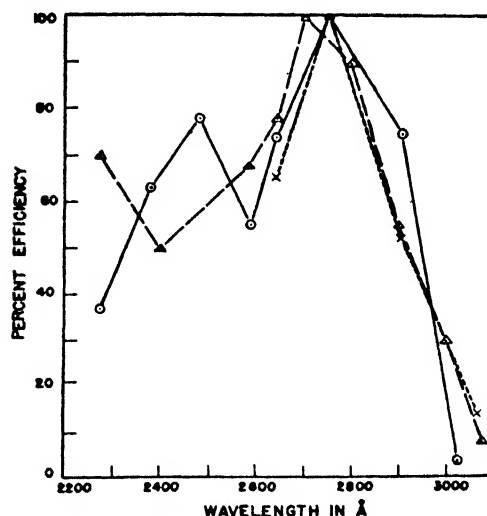


FIG. 6. Broken line: Relative absorption spectrum of sodium thymonucleate taking absorption at 2600 Å as 100 percent. Solid line: Relative effectiveness for mutation production in fungi. Dotted line: Relative effectiveness of mutation production for liverwort spores (Knapp, Reuss, Risse and Schreiber, 1939) taking the effectiveness at 2650 Å as 100 percent.

three curves have their major maxima around 2600 Å.

Wavelength dependence of mutation production for nine wavelengths between 2378 and 3022 Å has been studied for maize in a very thorough investigation by Stadler and Uber (1941). These authors irradiated the pollen under carefully controlled conditions. The absorption spectrum of the pollen wall and cell contents were determined. The wavelength

TABLE 3.* EFFECT OF MONOCHROMATIC ULTRA-VIOLET RADIATION ON THE SPORES OF *SPHAEROCARPUS*

Wavelength in Å	Relative percentage of sporangia setting	Relative percentage of mutations taking number of mutations at 265 mμ as 100 percent
2540	24.5	66.6
2650	5.7	100
2805	9.54	52.4
2973	111.4	13.8
3020	118	13.3
3130	116.5	0
Control		0

* After Knapp, Reuss, Risse; and Schreiber, 1939.

dependence of mutation production corrected for the non-nuclear constituents shows a definite maximum at 2537-2650 Å. Extensive work on the effect of ultraviolet radiation on the chromosome structure of *Tradescantia* has been reported by Dr. Swanson (1940). No wavelength dependence curves have been reported.

It has been known since 1930 that it is possible to produce mutations by ultraviolet radiation in *Drosophila*. An extensive investigation was reported by Mackenzie and Muller (1940). These authors used filtered radiation which separates certain regions between 2800 and 3650 Å. They reported efficient mutation production by regions around

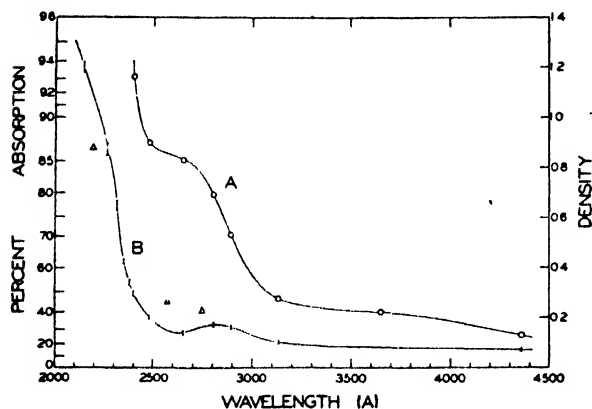


FIG. 7. Absorption curve of abdominal wall of *Drosophila melanogaster*.

(A) Absorption curve of wall with some tissue attached.
(B) Absorption of abdominal wall with tissue removed (Durand, Hollaender and Houlahan, 1941).

3100 Å. Work conducted in cooperation with Dr. Demerec (Demerec and Hollaender, 1940) with monochromatic radiation, shows a fairly high effectiveness around 3130 Å. Shorter wavelengths also will produce mutations but a large percentage of the flies either die or are sterile from the radiation effects. *Drosophila* sperm was irradiated in both these investigations when still present in the testis. The flies were pressed between quartz plates and the ultraviolet had to penetrate the abdominal wall, the testis tissue, and probably some storage tissue. The abdominal wall itself does not absorb highly at most of the wavelengths tested as the absorption spectrum given in Figure 7 shows (Durand, Hollaender and Houlahan, 1941). No exact data are available for the other tissues. This work is still in progress.

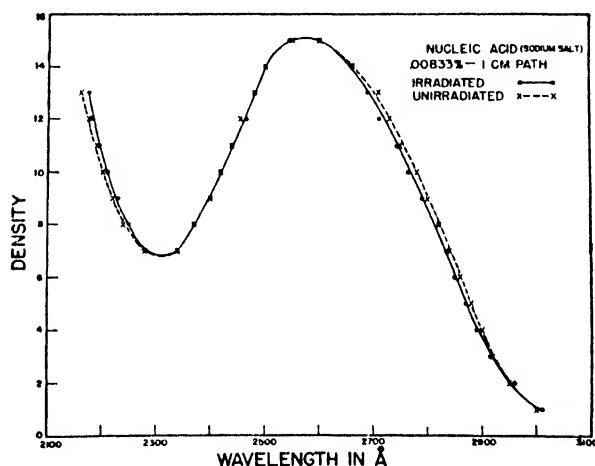


FIG. 8. Absorption spectrum of sodium thymonucleate in water solution before and after irradiation with 2537 Å (Hollaender, Greenstein and Jenrette, 1941).

It appears that wavelengths below 3000 Å cannot be readily tolerated by animal tissue, since a high percentage of the flies are not fertile after irradiation with short ultraviolet, and the finding that 3130 Å will produce mutations is a verification of the fact that toxic action and mutation production do not coincide at longer wavelengths.

METHOD OF ACTION OF ULTRAVIOLET RADIATION

Very little is known about the actual function of ultraviolet radiation in living cells. The fact is outstanding that for incident energy the 2600 Å region is the most effective one in producing toxic and genetic effects. This is the region which is most highly absorbed by nuclear proteins, especially nucleic acid. It is also known that microorganisms irradiated with moderate quantities of ultraviolet radiation will stain like living cells, despite the fact that they are unable to multiply. We have irradiated, in cooperation with Dr. Greenstein (Hollaender, Greenstein and Jenrette, 1941), sodium thymonucleate in water solution. This compound in water

forms a heavy viscous liquid. The stream double birefringence and structural viscosity can be determined readily. It was found that after irradiation with 2537 Å, the stream double birefringence and structural viscosity would decrease rapidly. It takes about 5.6×10^8 quanta per molecule¹ to decrease the structural viscosity by 10 percent. At the same time it is not possible to recognize any change in the absorption spectrum of the irradiated sodium thymonucleate nor any chemical change (see fig. 8). The action of the ultraviolet on the sodium thymonucleate is apparently a depolymerization. (For more detailed discussion, see paper by Greenstein.) Judging from our experiments on the irradiation of sodium thymonucleate *in vitro*, this compound could be changed physically quite readily inside the chromosome. Change of viscosity of the thymonucleate could produce a weakening of the carefully balanced structure of the chromosome which could lead to changes in chromosomes not readily recognizable in microscopic examination.

It is probably somewhat dangerous to overemphasize the importance of nucleic acid in the study of radiation effects on living cells. It is very well possible that in radiation produced mutations, the nucleic acid is only the "absorbent" agent, then transfers the absorbed energy to the protein closely associated with it. It is possible that among others, the following changes take place: 1) the breaking down of the nucleic acid; 2) the breaking down of the protein part of the nuclear protein; 3) a disruption in the relation of the nucleic acid to the protein, and finally, a combination of these three effects. We know at present too little about radiation effects to distinguish between these possible functions of ultraviolet radiation. We believe that more detailed knowledge of the structure, the chemical compounds, and physical organizations of the cell, especially the nucleus, may lead us to a more balanced interpretation of the functions of radiation and mutation production.

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¹ This value gives only the order of magnitude of the energy.

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DISCUSSION

DARBY: What is the purity of the band at 2650 Å?

HOLLAENDER: Quite pure, perhaps $\frac{1}{2}$ percent of 2537 Å.

DARBY: Do I understand that 2650 is your most effective wave length?

HOLLAENDER: This is the most effective wave length used.

MULLER: Do you think it possible that lack of rise in mutation rate with higher dosage is due to differential after effects of radiation on survival of mutated and non-mutated types? Could you test this by making a mixed colony of groups of spores of normal individuals and of mutants derived from earlier irradiations, and then compare, some time after irradiation of this mixture, the numbers of mutants surviving with those present originally?

HOLLAENDER: I believe that the decrease of mutation rate with high dosage is caused by a combination of effects. Attempts to check with the test you suggest has given no clue, since normal spores grow in general much more rapidly than irradiated spores. Most of the mutants have slow growth rates.

ZAMENHOF: If one irradiated a small spot of a fungus spore, one could determine the distribution of chromatin in fungi. We could deduce from this the sensitive volume in fungi.

HOLLAENDER: Cole has studied the absorption spectrum of different areas in spores and finds three regions highly absorbing around 2650. Cytologically there is a single nucleus. It would be extremely difficult to irradiate such a small area in a fungus spore. The total diameter of a spore is about two to four micra.

Investigations of tobacco mosaic virus give a small maximum of sensitivity at 2650 Å, and higher sensitivity at shorter wave lengths.

JONES: The frequency of spontaneous changes increases with age. Is irradiation accelerated aging?

HOLLAENDER: No quantitative analysis of the number of mutations produced by aging has been made. In general, fungus spores are better suited for study of the effects of chemical or physical agents than eggs or sperm.

Sensitive volume discussions help us very little

toward understanding the effects of ultraviolet radiation on the microorganism.

UBER: How pure is the radiation at 2950 Å?

HOLLAENDER: The line which I called 2950 is the line 2937 of the mercury spectrum. This line comes through our monochromator in fairly good purity.

The absorption spectrum of nucleic acid has been determined only for wave lengths of up to about 3100 Å. We have found at wave lengths longer than 3000 Å several interesting effects in spite of the fact that no measurable absorption has been found in this region of the spectrum. Absorption spectra have an error of about 5 percent, and it is possible that 0.1 percent absorption may be sufficient to account for the effect we have found at wave lengths between 3000 and 4000 Å.

DELBRÜCK: A remark concerning the quantum yields of effects of ultraviolet radiations.

1) Local and gross yield. The quantum will be absorbed by some chromophore group of the gene, virus or enzyme and in a fraction of cases the absorption will lead to a chemical reaction which is injurious to the function of the group or of a close neighbor of it. This fraction we will call the *local quantum yield*. Observation will give the ratio of the inactivated fraction to the number of quanta absorbed per molecule. This we will call the *gross quantum yield*. The local yield cannot be calculated from the observations. It will differ from the gross yield for several reasons.

a) The molecule will contain other chromophore groups which will also absorb light, but the absorption will not be followed by an injurious chemical reaction. In this case the local yield will be n_a times greater than the gross one, if n_a is the ratio of insensitive to sensitive chromophore groups. This factor will on the whole be the same for all sizes of molecules, but it may differ widely in individual cases, and will also be different for different classes of molecules. For instance, it will be different for proteins and nucleoproteins, if the nucleic acid should just happen to add insensitive chromophore groups in the nucleoproteins.

b) The activity of the molecules may reside in several active centers, so that elimination of one center will only eliminate a fraction of the activity of the molecule. In this case (enzymes), the local yield will be n_b times greater than the gross one, if n_b is the number of independently active centers. This factor will be the greater the greater the molecule, since it is probable that larger enzyme molecules have more active centers.

c) The activity of the molecule may depend on the intactness of every part of it, so that an injury to any part of it will eliminate the activity of the entire molecule. In this case (genes, viruses, phages) the local yield will not be shifted systematically, but the gross yield will represent the arithmetic mean of the yields of all possible modes of inactivation and may not coincide numerically with any one of them.

For these reasons the gross yields can at best only

give an indication of the order of magnitude of the local ones, and they are not comparable quantities for materials which differ in composition (proteins and nucleoproteins), or in type of inactivation (enzymes against viruses etc.).

2) Wave length dependence of the yield. The gross yields in the cases of urease and of pepsin are quite small and in the case of urea the yield is nearly independent of the wave length in the region of the absorption bands belonging to the first electronic excitation of the ring compounds. It seems probable also that the local yields are smaller than unity. That means that the probability of the occurrence of the injurious chemical act is independent of the amount of vibrational excitation of the absorbing molecule. The explanation is that the conversion of the excitation energy into the chemical energy is a slow process, i.e., slow compared to the time it takes to dissipate the vibrational energy, about 10^{-12} seconds. It must also be slow in comparison to some deactivating process with which it competes, and which wins out in the large majority of cases, hence the small yields. The deactivating process may be fluorescence radiation, or a radiationless transition to the ground state.

The point I want to make is this: the independence of the yield on the wave length shows that the secondary chemical act is a slow process, and the smallness of the yield also shows that the chemical act is slow compared to some deactivating process. These two observations therefore fit together. They also fit with the idea that the chemical act does not concern the ring part of the side chain but a peptid bond in another part of the same molecule. Such a transfer of excitation energy to a distant part of the molecule depends on the interaction of several types of vibrational motion and is quite generally a slow process. It is very fortunate that we know from Carpenter's experiments that a transfer of excitation energy from the ring to the peptid bond is possible. (Carpenter, D. C., 1939, SCIENCE 89: 251.)

UBER: With reference to the quantum yields for the substances just mentioned in this discussion by Delbrück, the experimental data are too meager at present to permit an evaluation of the various suggested possibilities. The gross yields are small for urease and tobacco mosaic virus; the yield for pepsin is somewhat greater and our preliminary data for trypsin indicate a value of about 0.02 for inactivation. Considering the molecular weight of trypsin, the latter value is not particularly small. The question of wave length dependence requires more experimental attention, but it seems evident from existing studies that no great variation in yield within the range corresponding to a particular electronic transition is to be expected, except in cases where two such transitions overlap. In the paper of Carpenter just referred to by Delbrück, mention is made of a threshold for stearic anilide in the center of such an electronic absorption band. So far, this remains unexplained.

MUTATION IN DROSOPHILA, BACTERIA AND VIRUSES¹

JOHN W. GOWEN

We speak of the sudden appearance of an individual *Drosophila* showing a characteristic markedly divergent from the wild type as a mutation. Bacteriologists and pathologists referring to similar changes in bacteria or viruses use the term variant and avoid the term mutation. Both groups usually define the term mutation as a relatively permanent heritable change in the organism. Virus changes are regarded as less permanent than changes in higher forms. The particular variant may revert to the parent type. Growth on culture media, in animal passage, etc. is said to facilitate these changes. Bacteria may be "trained" to take on other characteristics. To the bacteriologists the changes are not as permanent in the genetic sense as are mutations in higher forms like *Drosophila*.

Superficially the observed changes do suggest lability in the variants of single celled organisms. The investigator may introduce a single celled culture composed entirely of rough bacteria into a host and in a week or less recover only bacteria having smooth colony characteristics. The change is sudden and nearly 100 percent complete. This smooth colony type organism under other circumstances may change equally abruptly back to the rough form. The condition necessary to facilitate the appearance of these variants may be controlled to some degree. With these results it is not surprising that changes of media have been interpreted as causative in initiating variants rather than as correlative, and have thus lead to avoidance of the mutation concept as sufficient to account for the variants observed.

In evaluating this conclusion there are some marked differences in our ability to observe mutation in the higher and lower forms of life as well as great differences in the observed effects of given selection pressures which need to be taken into consideration. In 24 hours many bacterial species will reproduce to such numbers that if their rate of mutation is comparable to that thought true for *Drosophila*, each gene the bacteria possesses should mutate at least once. With even slightly favorable selection, replacement of the parent population by the mutant is easily possible in a short interval of time.

As these concepts affect our understanding of inheritance generally and the gene in particular as well as any significance we may attach to the host-gene, pathogen-gene relation in disease, our laboratory has investigated mutation rates in the three organisms—*Drosophila melanogaster*, tobacco mo-

saic virus and the bacterial corn wilt disease *Phytophthora stewartii* under as nearly identical conditions as the differences in the material and the exigencies of the situation would admit. Associated with me in various phases of this work have been Dr. H. C. Fryer, Dr. R. E. Lincoln, Dr. M. R. Zelle and Mr. G. W. Kohler.

If we assume that the basic element in the phenotypic variation of these three forms are changes in gene-like structures then we would expect that under like stimuli the reorganizations accomplished would be comparable. That all these reorganizations would be observed is doubtful. In *Drosophila* the chromosome matrix is certainly an important element in keeping the different genes in groups. The development of the gene's characteristics depends on the union of a compatible sperm with a suitable egg. The cytoplasm is important and may carry along genes which on their own would fail to develop. Moreover the double sets of genes aid poor genes to reproduce. The end product of the gene's action is the unique configuration of cells which we call the character.

Chromosomes in the bacteria are not proven. Whether the genes are in diploid, haploid or some other multiple is unknown. If the situation is as in some fungi the nucleus would probably be haploid but the cell might contain more than one nucleus. With reproduction these nuclei may multiply differentially and migrate irregularly from cell to cell. The character in bacteria as in *Drosophila* is the resultant of controlled cell configurations.

The viruses differ from *Drosophila* and bacteria in requiring the host chemical environment rather than its own cytoplasm to develop the character by which we classify it. The lack of a supporting material around the virus particles would make it not improbable that many configurations in their structure would be lethal. Similar changes might survive in such forms as *Drosophila* or bacteria.

In each of these materials special variants relations have been chosen for study. Each character contrast is known to be relatively stable at both ends of the scale. Thus the aucuba form of tobacco mosaic virus may change to the ordinary form and be essentially stable after the change. The ordinary form may change to the aucuba and become stable.

The following mutant genes and their wild types were chosen for study in *Drosophila*: scute, echinus, cross-veinless, cut, vermilion, forked, carnation, aristaless, dumpy, purple, curved, plexus, speck, roughoid, hairy, scarlet, striped, sooty and claret. When possible each mutant was tested for identity with the suspected gene. These verified mutant

¹ Journal Paper No. J-900 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 573.

changes represent gene mutations or small non-lethal deficiencies giving the recessive characteristic.

The contrasting characters in the bacteria were yellow *vs.* white colony color; smooth *vs.* rough colony surface; normal *vs.* small colony size. Each of these characters may have more than one distinguishable self-perpetuating type. These characteristics are the typical variant types recognized in the bacteria. They cannot be proven to be unit gene changes when they mutate although, judged from the *Drosophila* work, the probability is high that they are.

Two variant types of tobacco mosaic have been studied. The first appears as necrotic lesions on *N. sylvestris* where the parent type shows only mottled lesions. The second shows mottled lesions on *N. sylvestris* where the parent type shows discrete necrotic lesions. The parent types producing these

This method of adjusting the dose is not particularly satisfactory with *Drosophila* and bacteria as depth of the containing materials cannot be adjusted to a sufficiently thin layer.

NATURAL MUTATION RATES

Mutative changes, even at a small rate, offer particular problems in viruses and bacteria as contrasted with higher forms in which there is a mechanism to hold the dividing cells together. If a *Drosophila* male has a mutation in the early germ cells which through multiplication reaches the sperm cells it may be detected as an early mutation with subsequent division due to identical mutant sperm tracing to that male alone. With viruses or bacteria the case is different. The mutant occurs, separates from the parent, continues to reproduce its kind, separates and mixes at random with the type form.

TABLE 1. FREQUENCY OF ALTERNATIVE CHARACTERS IN PARENT STOCKS UNDER NATURAL CONDITIONS

Parent culture	Number examined	New type	Ratio $\times 10^{-4}$
<i>Virus</i>			
Ordinary mosaic	649,307	975 aucuba	15.0
Aucuba mosaic	198,965	295 ordinary	14.8
<i>Bacteria Ph. stewartii</i>			
Dark yellow, rough 1 medium sized colony	1,524,000	92 color smoothness size	0.70
<i>Drosophila</i>			
Wild type	105,916	5 any observable changes	0.47

effects are ordinary tobacco mosaic and aucuba mosaic, the names which will be used to designate the phenotype of any variant observed. These three species have been studied for their natural rates of change from one type to the other and also for changes in these rates with exposure to radiant energy.

These materials have been treated with X-rays² of three different energy levels, ca 6.0, 8.2 and 17.6 KV, as determined by absorption measurements of the radiation in aluminum. The X-rays were from X-ray tubes with targets of chromium filtered through aluminum, of copper filtered through nickel and silver filtered through palladium, the thickness of the windows being adjusted to absorb about 50 percent of the initial radiant energy. The beam intensity at the surface of irradiation was measured by an ionization chamber designed by Mr. Pinney after the general plan of Taylor and Singer (1936). The ionization readings for the different wavelengths were corrected to standard conditions of temperature and pressure. Further correction was made for the absorption in passing through the material in which the sperm, bacteria or virus was irradiated, linear and mass absorption coefficients for each material being experimentally determined.

The numbers of the mutant may increase, and depending on the reproducing and selecting differentials, may materially affect the proportion of type to mutant cells. As these variant types cannot be distinguished from those due to individual mutation it follows that natural mutation rates dependent on mass sampling may be in great excess of the true rate. As even single celled cultures growing to usable sized cultures of 0.5 to 10 billion or more organisms are equally subject to this difficulty, it follows that a determination of mutation rates for these forms requires special treatment. Broth tubes were inoculated with a culture from a single colony of apparently pure type. After suitable growth the bacteria from a tube were plated and counts made for variants. Like variants from single tubes were regarded in two ways; either as representing a separate mutation or as the repeated types representing but one single mutation with subsequent reproduction. Rates calculated by the first hypothesis lead to a maximum value for mutation, those by the second to minimum values.

We have no extensive natural rates of mutation of our own for *Drosophila* genes. In fact rates for the individual visible gene scarcely exist. The nearest approach to natural rates are the controls used for experiments on the relation of heat to mutation rate of Plough (Plough and Ives, 1935) and others. These rates are for all conceivable visible gene changes in the fly. The mutation ratio thus obtained

² I am deeply indebted to the Fuller Fund and the International Cancer Research Foundation for cooperating in these investigations by supplying this equipment.

is too large by a multiple of the number of genes involved.

Before using the ordinary or aucuba forms of the tobacco mosaic viruses as a stock material, each virus was passed through eight successive *N. sylvestris* plants. The strains used showed no lesions of the contrasting type during all of these eight passages. Further multiplication of the particular virus took place in *M. tabacum* plants which, besides showing no lesions and isolated from all other plants, had been tested for the presence of virus.

The experiments are designed to ascertain the rates of change of the aucuba parent to the ordinary mosaic variant and also the ordinary mosaic parent to the aucuba variant.

For the aucuba parent, the numbers of virus particles spread on the *N. sylvestris* leaves were estimated by counting the aucuba lesions appearing on the treated leaves. The ordinary mosaic mutants appear as mottled types on the new leaves.

The particles of the ordinary mosaic parent spread on the *sylvestris* leaves were estimated by tests on 16 bean leaves where this virus produces necrotic lesions equal to about 25 *sylvestris* leaves (ca 92 percent). The variants appear as necrotic lesions on the *sylvestris* leaves. An error is involved in the variant rates as determined from the aucuba parent to the ordinary mosaic type as it is impossible to distinguish plants where only one virus particle mutated from those where two or more particles change.

Each parent type shows aberrant forms. The number of these off types is 25 times larger for the characters studied in the viruses than for those in either the bacteria or *Drosophila*. There are several possible explanations for this difference, one of the most obvious being contamination. Contamination seems unlikely on the following grounds. The material involves more than 150 experiments, repeated in various ways and at different times. The different experiments are concordant in showing a low but persistent proportion of the unexpected types. The precautions taken to insure the purity of the parent virus types appear adequate. Both types of virus show some of the other type on plating out whereas only one type of virus has been used at any one time. Untreated plants have not shown the lesions although distributed at random among the test stock. Several of the mutant type show slight and persistent differences from each other and the parent form.

A possible explanation to account for the presence of these new types as mutations would be that they are either taking place at a higher rate than in the other forms studied or, perhaps more likely, they are mutants which have some early selective advantage, and differential multiplication.

The aucuba virus changes to the ordinary type with about the same frequency at which the ordinary virus changes to the aucuba type.

The data of Lincoln and myself on *Ph. stewartii*

show that the dark yellow, rough I, medium sized colony forms may change in any or all of these particulars even though the particular organism is derived from a single cell. As Lincoln (1940) has shown these changes are also reversible in most instances. Thus pale yellow rough forms may mutate to the dark yellow, or to white, to other rough types or to smooths and to types with a different growth control leading to colonies of other types. The white form does not change readily to yellow types although it may change to bacteria displaying other colony surface characteristics. The rates at which these different parent types form new types varies with the particular parent type. The range of variation is from 1 in a million to 1 in three thousand.

The new types observed in *Drosophila* are less frequent than those in either the bacteria or the viruses. The rate is based on the organism as a whole rather than on the gene loci capable of mutation to visible forms. The rate of observable changes per gene locus would be at least a fiftieth of this tabulated rate. However, not all forms are equally detectable, a fact which appears reflected in the data as they emphasize recessives which are sex-linked and dominant.

X-RAY STIMULATION OF MUTATION

The question arises, are these changes observed in the viruses and bacteria, mutations or do they have some other characteristics? The suggestion that they are similar to the mutants of *Drosophila*, at least as far as the bacteria are concerned, is seen in the rates of natural change. A further indication of the similarity would be a like behavior of the three species when exposed to an agent capable of accelerating mutations. X-rays of low kilo voltage have been used for this purpose. The radiation on which chief emphasis has been placed is that from

TABLE 2. AUCUBA MOSAIC LESIONS ARISING FROM ORDINARY MOSAIC VIRUS TREATED WITH X-RAYS

Roentgens of exposure	Ordinary mosaic lesions on beans	Aucuba lesions on <i>sylvestris</i>	Ratio $\times 10^{-4}$
control	225,750	179	7.9
149,000	161,737	527	32.6
248,000	29,268	157	53.6
490,000	4,052	28	69.1

TABLE 3. ORDINARY MOSAIC LESIONS ARISING IN AUCUBA MOSAIC VIRUS TREATED WITH X-RAYS

Roentgens of exposure	Aucuba lesions	Ordinary mosaic lesions	Ratio $\times 10^{-4}$
Control	198,965	295	14.8
62,500	111,493	233	20.9
132,000	103,704	446	27.2
438,000	43,755	108	24.7
748,000	12,041	55	45.7
1,355,000	3,720	33	88.7

a copper target with an incident wave length of 1.5 Å.

The data of Table 2 show a slight but persistent increase in the number of new forms as the X-ray dosages increase. A similar trend occurred in those experiments in which the aucuba virus was used as the parent type, Table 3.

Graphs showing the trend of these changes are presented in Figure 1.

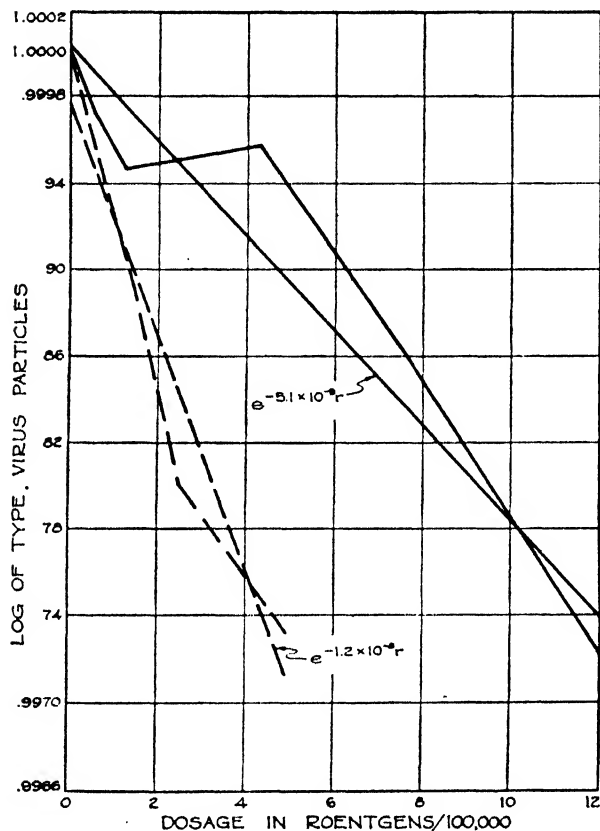


FIG. 1. Effect of X-rays on aucuba or ordinary mosaic particles. Solid line: log of virus particles where parent type is aucuba and unexpected type ordinary. Dotted line: parent type ordinary, unexpected type aucuba.

Both curves in Figure 1 show an increase in the proportion of variants with increasing X-ray dosages. The increase in the aucuba to ordinary direction is about half that in the reverse direction. Part of this difference is accounted for by the fact that two or more changes in the same plant are indistinguishable from one change whereas all the aucuba changes have the possibility of expressing themselves.

The rate of change in either virus particle per roentgen is small. This corresponds with the small size of the particle as the X-ray target. The easiest change to make in the virus particle is that leading to its inactivation. Changes leading to visible mutations are made with more difficulty. This result

corresponds to our finding on *Drosophila* genes.

In the data of Lincoln and Gowen on the rate of mutation in *Ph. stewartii* 310 mutant forms were observed in 385,919 colonies. The different experiments had a range in survival value from 252 to 37,000,000 per 100,000,000 initial organisms. At the point where 8,000 organisms survived out of the initial 100,000,000 the rate of mutational change was 4.2 per 1,000 colonies. From extensive experiments on survival of these bacteria under X-rays of this wave length this survival percentage corresponds to a roentgen dosage of roughly 38,000 r incident to the bacteria. The average rate of visible mutation per roentgen per bacteria corresponds to 1.15×10^{-7} .

Experience has shown that three major characters were emphasized in determining the frequency of these changes: the color of the colony, the colony surface and colony size. The mutation rate per locus studied would be at least one third less than the 1.1×10^{-7} given above. For purposes of this paper we shall consider the rate of change of these bacteria under X-rays as 3.7×10^{-8} per roentgen per character.

In their work on the effect of X-rays of 6.0, 8.3 and 17.6 KV Fryer and Gowen found 49 verified visible mutations in 168,945 gametes treated with roentgen dosages between 2475 and 7250 units incident to the sperm of *Drosophila*. The rate of change per roentgen per gene followed was 6.96×10^{-8} . Besides these verified mutations there were other mutants of like phenotypes which were sterile. Such types indicate a mutation of the irradiated wild type allele to the recessive type or a deficiency in that region. The total of these untested variants plus the verified gene mutations was 123 or the rate of observed change was 1.7×10^{-7} per gene per roentgen. This rate seems high. For the forked, miniature and eosin loci the data collected by Timofeeff-Ressovsky (1937) indicate rates of 6.6×10^{-8} , 2.4×10^{-8} and 2.6×10^{-8} . The data of W. G. Moore cited by Johnston and Winchester (1934) for sex chromosome genes gives a higher figure of 48 in 116,200 gametes at a dosage of 3,975 r or a ratio of 1.04×10^{-7} . However, the citation may possibly be a misinterpretation as what appears to be the same but much abbreviated data in Moore's paper of 1934 gives 33 changes in 116,200 with a dosage of 1,325 r or a rate of 2.1×10^{-7} per roentgen per gene.

For mutations away from the recessive type Johnston and Winchester list 713,001 tests on 10 sex linked genes. Twenty-four mutations were observed of which 12 proved sterile and 12 were verified changes of the type suspected. The rate of these changes was 8.5×10^{-9} . Timofeeff-Ressovsky lists tests on 390,709 genes in the sex and third chromosomes. These genes were exposed to 4,800 r. Twenty-six changes were observed or a rate of 1.4×10^{-8} per roentgen per gene.

The results for the ratio of change observed in

the different species under X-ray may be summarized.

Tobacco mosaic virus particle	mutations/roentgen/	1.2×10^{-8}
Tobacco aucuba virus particle	mutations/roentgen/	5.1×10^{-9}
<i>Ph. stewartii</i> mutations/roentgen/character		3.7×10^{-8}
<i>Drosophila</i> wild type genes mutations/roentgen/gene		1.0×10^{-7}
<i>Drosophila</i> mutant genes mutations/roentgen/gene		1.2×10^{-8}

The rates of change of each of these self reproducing entities are strikingly alike. All of these substances are affected at comparable rates by the radiant energy of the X-rays. As this agent is credited with producing mutations in many higher forms it is certainly no stretch of the same reasoning to attribute X-rays with producing like results in the lower forms. Under certain selective agencies these variants may replace the type form, may revert to the type form and be replaced by the reversion or do all of these things rather rapidly, but when the rate of reproduction is considered they do these things no more readily than other higher forms where the variants are accepted as mutations dependent on genic substances. In view of these facts the results of these experiments indicate a common mechanism for perpetuation of the inheritances in all groups from the highest to the lowest. The basis for bringing about change in the inherited characteristics of the different groups appeared to be similar in each group.

Mutations from wild type genes to other alleles seem to be more frequent in *Drosophila* than reverse mutations. On this basis it might be argued that in nature where two types are present the so-called wild type can be established by its ease of mutation. The ordinary mosaic has the greater rate of change. On this ground the ordinary mosaic form of the virus would correspond to the wild type. Aside from the somewhat tenuous reasoning involved there are certain facts of experimentation which make this conclusion appear doubtful. As pointed out earlier the aucuba mutant may be detected when it appears. Two or more ordinary mosaic mutants could not be detected in the same plant. This fact does not account for all of the differences observed between the two strains but does tend to draw the rate of mutation for the two groups closer together.

The mutations in the viruses have been classified in alternate classes as though the changes were all identical. In large part they are all of either one or the other type. But work with a large number of these similar forms leads to the impression that small differences in their effects exist. These differences are often not classifiable and overlap as between different mutants. They suggest possible allelomorphous arrangements comparable to those found in the different series in *Drosophila*. Similar differences are seen in the characters appearing as the result of mutation in *Ph. stewartii*. The rough series, for instance, varies by discrete steps from

a very smooth colony to a markedly rough mutant. Mix these colony types together, or have them appear all at once, and it would be very difficult to separate them. However, have them appear as descendants of single mutated bacteria and their minor variations are classifiable. The same is also true of the mouse typhoid organism, *Salmonella typhimurium* as shown by Zelle (1940) in our laboratory.

These changes affect the whole picture of disease and the epidemic process. The mutants of the viruses make their attack and express their symptoms in distinctly different ways. They are markedly different in their pathogenicity. Virulence differences also exist for some but not all of the mutants in the *Ph. stewartii* and *S. typhimurium* bacteria. The direction of the change is not predictable as the virulence may increase with one change, remain the same with another and decrease with a third, the phenotypic change of the colony in the particular mutants remaining indistinguishable.

The mutation rates of these different substances are nearly comparable if account is taken of the number of characters involved in the study. The inactivation rates are not so alike. The inactivation rate of a culture of the tobacco viruses is 5.8×10^{-6} per roentgen per particle. The inactivation rate of *Drosophila* sperm as expressed in the incapacity of the egg fertilized by irradiated sperm to hatch ranges rather widely, but for present purposes will be assumed to be 2.9×10^{-4} . Similarly the difficulties in measuring roentgen dosage for bacteria suspended in broth solution are such as to make the following measurement subject to revision but it may be assumed for the purposes of the discussion to be 2.5×10^{-4} . The differences in these rates, as we have shown elsewhere for many other species, are in order of the sizes of the exposed entities, the viruses inactivating less readily than the bacteria or *Drosophila* sperm. Not all of the absorbed energy is converted into inactivation, however. A fraction of this left over energy is evidently utilized in mutational rearrangements of the substance to produce entities developing other phenotypic effects than the parent forms from which they arose. Instead of being inactivated these forms retain their self reproducing role.

If we compare the energy for inactivation with that for mutation of the virus we find that one hundredth or less of the energy absorbed goes into mutation in contrast to that which expresses its action in inactivation. This may mean that only a relatively few of the energy bonds holding the molecule together may be shifted, thus producing a mutation, and leave the reproductive property uninjured. Or put in another way we might say that more of the molecular arrangement is significant to reproduction of the molecule than to the phenotypic expression of the character by which we classify the virus as ordinary mosaic or as aucuba. In our studies several other mutant types have appeared for which the same conclusion would be true al-

though the technical difficulties in handling them are such that we have not as yet the required quantitative evidence.

If we assume that inactivation or death in eggs fertilized by irradiated *Drosophila* sperm is due to dominant lethal mutation in a narrow sense then the inactivation rates of each group should be in proportion to the genes capable of mutating to these dominant lethal effects. The rates of inactivation of sperm and bacteria are comparable. On the above basis the dominant lethal genes should be comparable in numbers in the two species. We are, I presume, too egotistical to accept the view that the number of genes required to organize a bacterium are as great as those for a fly. Nor is it necessary to accept this view.

If bacteria are like the fungi with the vegetatively reproducing parts haploid, the dominant is not expressed more frequently than the recessive. Under these conditions the recessive lethal changes would express themselves as non-reproducing bacteria as do dominant lethal changes in *Drosophila*. The proportion of inactivated bacteria would be much greater than that noted for the diploid *Drosophila* eggs. This reasoning shows that we might expect a greater inactivation of the bacterial cells carrying less genes than *Drosophila* eggs.

On the same reasoning the genes vital to reproduction in the bacterial cells should be comparable with the virus molecules in their inactivation rates. A change leading to the inability of a single one of these genes to reproduce would eliminate the affected cell. Under these circumstances the mass of gene substance in the bacteria comparable to that in the virus would be proportional to the inactivation rates or about 50 times the volume of substance necessary to reproduction that is found in the tobacco mosaic virus.

SUMMARY

This paper presents evidence on the spontaneous frequency of variants in viruses, bacteria and *Drosophila*. The rate of spontaneous variation is shown to be small.

The frequency of variants in the three forms is increased when they are exposed to comparable X-ray dosages. The rates at which variants appear are of the same order of magnitude. This evidence is interpreted as indicating a common basic structure for inheritance in each group, the changes of which are in the nature of mutations.

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DISCUSSION

(Some of the discussion was combined with that of the paper of Muller.)

UBER: As to the size of the sensitive volume, or what I should prefer to call the radiochemical field per ion pair, for the inactivation of this virus protein, you reported a tentative value of approximately 0.025, at Woods Hole last summer. Do you still hold to that value or has it been revised in line with more recent data?

GOWEN: The data presented here are of course for mutation and not inactivation. The inactivation rates are an integral part of these results, however.

UBER: This result may be compared with that of Luria on the inactivation of bacteriophage by X-rays. He obtained a value of approximately unity, but on the assumption that an ion cluster was responsible for the inactivation.

For comparison with these yields per ion pair with X-radiation, I should like to mention the corresponding quantum yields with ultraviolet radiation, which it has been possible to calculate from the data of Price and Gowen. Various approximations were made, due to the fact that monochromatic radiation was not employed, but the resulting value is probably of the correct order. It is 0.000026, and has been confirmed satisfactorily in a recent personal communication from Lea, who reported a value of 0.000070 at λ 2537 Å.

GOWEN: The size determinations depend quite largely on your concept of how X-rays act. In a period of 30 years of development, these ideas have changed from a point hit theory.

UBER: Can the figures of the size of virus particles depend on the molecular weight?

GOWEN: Yes, the specific gravity is considered important by all investigators, I understand.

The determination of size from X-ray measurement would depend on what physical hypothesis you use, of which one would be the hypothesis of ion pairs acting in cluster averaging three.

STANLEY: In the first sedimentation studies on purified tobacco mosaic virus Eriksson-Quensel and Svedberg arbitrarily assigned an asymmetry constant of 1.33 and calculated a molecular weight of 17 million. Since then the asymmetry constant has been determined to be about 2.34 and using this value and the original value for the sedimentation constant the calculated molecular weight becomes about 40 million. This value has since been confirmed by means of several different methods.

GOWEN: Lea and Smith have a more recent approach to the question of tobacco mosaic size, utilizing the ion pair cluster effect.

FANKUCHEN: The X-ray diffraction pattern has been used to give the dimensions of the virus cross section.

LURIA: Lea's experiments and also some of Gowen's give a sensitive volume corresponding to about 35 million molecular weight, in good agreement with Dr. Stanley's data.

STANLEY: Did you use the virus in the living leaf in these experiments?

GOWEN: I have done this, but the data presented here are from dried virus in thin layers.

STANLEY: Was the increase in the number of variants produced 10-fold, 100-fold or of what order of magnitude? Stubbe and collaborators reported irradiation experiments on purified preparations and concluded that they got an increased rate over the normal. Later they appear to have decided that the increase was not significant but then claimed a difference was established when they then used living leaves. Recently normal plants were irradiated and infected with virus 24 hours later; an increased number of variants were obtained. However, because of the small number of tests that were made, I doubt that the difference is significant. I should mention that under presumably normal conditions, the number of variants demonstrable depends on the time the virus is allowed to develop on the plant and is variable under normal conditions.

I might point out that the X-ray data indicate that the tobacco mosaic virus molecule consists of a series of subunits of dimension $68 \times 88 \times 88 \text{ \AA}$ which would correspond to a unit of about 500,000 molecular weight, while the molecular weight of the virus molecule is about 40 million. No one has demonstrated tobacco mosaic virus activity to be associated with any particle of less than 40 million molecular weight.

GOWEN: Our experiment covering a range of potential energies of 6 to 1000 kilovolts indicate a wave length effect.

MULLER: Would this affect the sensitive volume?

GOWEN: Yes, depending on where you took your rates.

MULLER: It is strange that this wave length effect is not obtained in *Drosophila*.

GOWEN: We performed two experiments using the same wave length in *Drosophila* as in the virus. The first seemed to indicate a wave length effect, the second more extensive experiment seemed to indicate none. The data were on the first and second chromosomes.

LURIA: In bacteriophage inactivation there is not a wave length effect between 17 and 1000 KV.

STANLEY: There is a deep-seated difference in the amino acid composition of the different strains of

tobacco mosaic virus. Since the strains presumably have a common origin our data would indicate that the formation of a virus of a given amino acid or even perhaps by the building in of an entirely new amino acid. It will be interesting to determine whether or not such a deep-seated change can be produced by X-radiation.

GOWEN: Of seven viruses studied, the inactivation rate was the same.

FANKUCHEN: A hit may affect the way in which the virus reproduces itself.

GOWEN: Ultraviolet might not have sufficient energy to carry over. Sommermeyer and Dehlinger think of the reorganization in terms of changes in crystal structure rather than chemical changes. No doubt there are differences between X-ray and ultraviolet action. Changing values on an erg basis, the relative levels are pushed way down. The per ergs effect differs greatly between ultraviolet and X-ray.

MULLER: With regard to Stanley's remarks that different proportions of amino acids emerge, the effect might be analogous to that in the Bar case, where there is removal of material from one place to another and then reproduction of the partially duplicated type.

GOWEN: I wonder if we should limit the term "duplication" to things added as extra physical entities rather than internal reorganization.

CHILD: In this connection we should bear in mind the results obtained in the use of isotopes in the study of intermediary metabolism. It has been shown very clearly that the molecules which make up protoplasm are in a continuous dynamic interchange with the molecules of their environment. Living things are not made up of the same molecules from one moment to the next. Whole amino acids may leave protoplasm in exchange for a similar unit from the external medium. If while these exchanges are occurring in a gene, that gene or part of it is ionized, it would be easy to imagine a gene reduplication of a slightly different type from the parent gene, that is, a mutation. Additions to the gene could readily occur. I hope that Dr. Rittenberg will tell us more about this possibility in his paper.

FANO: To consider ion clusters may be misleading, inasmuch as three ion pairs is the average but is not the most probable value. The most frequent case is one ion pair in a cluster.

IMAGE FORMATION BY ELECTRONS

V. K. ZWORYKIN

The formation of images by electrons, much as optical images are formed by light, has given rise to a new branch of science called "electron optics," which forms the basis for many interesting and important uses of electrons, including modern television and the electron microscope. As the determination of the paths of light rays constitutes an important part of the design of an ordinary optical system, the determination and control of electron trajectories is the function of electron optics.

Early studies of the behavior of cathode ray beams under magnetic and electric fields revealed that these rays consisted of particles, later to become known as electrons, which had a fixed ratio of charge to mass, and that the paths could be completely determined by the laws of ordinary mechanics. It soon became evident that not only could these cathode rays be investigated by means of magnetic and electric fields, but also that the converse was true, and that cathode ray beams could be used to measure these fields. While investigating cathode-ray tubes, made possible by this phenomenon, an interesting observation was made. It was found that by suitably adjusting the voltage on the concentrating electrodes, or on the magnetic focusing coils, a clear enlarged image of the thermionic cathode could be observed on the fluorescent viewing screen. These images, in fact, were frequently used to determine whether the activation of the cathode was uniform, and served as a convenient way of studying defects in the cathode. These tubes can be called forerunners of the electron microscope.

Towards the close of the nineteen twenties, the importance of electron imaging was beginning to be more apparent, and a good deal of work was being done to determine its nature. Theoretical and experimental studies published by Busch, Picht, Davission, Knoll, and others showed not only the possibility, but also the general practicability of electron lenses. By 1932 an electron optics based upon the analogy between light and electron motion had become a clearly recognized field.

Once the foundation of this field had been established, progress was rapid, and practical applications followed almost immediately. Systematized knowledge of electron optics could be applied directly to the problem of building an electron gun which was capable of producing the high density, fine cathode ray beam required in modern television and cathode ray tubes. The study of electron paths was applied to the problem of amplifier tubes and led to the development of the beam power tube, the

secondary emission multiplier, and other similar devices. At the same time much work was done on extended electron images. It was found, for example, that sharp, undistorted electron images could be reproduced from optical images focused on a photoelectric cathode. Another important application of electron imaging was in the electron microscope. The first compound microscope employing electrons as the imaging means was reported by Knoll and Ruska in 1932. The development of the electron microscope has progressed rapidly and today it has become a practical research tool, capable of resolving objects at least fifty times smaller than can be seen with the best optical instrument.

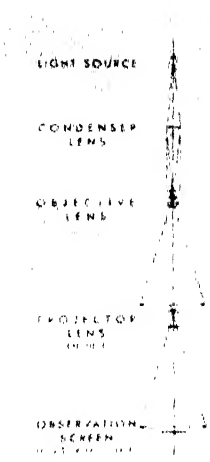
In principle the electron microscope is very similar to the conventional light microscope. Figure 1 shows schematically a comparison between the fundamental elements making up these two types of instruments. Each microscope has a source of the radiation used for making observations, condenser lenses for concentrating the radiation onto the specimen, an objective lens which forms an enlarged first image of the specimen, and a projection lens which forms the final image. In the electron microscope the source of radiation is a thermionic cathode in an electron gun. Electrons from the source are accelerated to a high velocity as soon as they have left the cathode. The electron lenses may be either electrostatic or magnetic, and comparable results have been obtained with both types of systems. However, so far, most electron microscopes in use at present are equipped with magnetic lenses because of practical considerations.

The process by which the enlarged first image is obtained is closely related to the phenomenon of diffraction of either light or electrons, and consequently dependent upon the size of the object being imaged, the angular aperture α of the lens, the wavelength λ of the radiation employed, and the index of refraction μ of the medium surrounding the object. The following expression gives the size of the smallest detail which can be resolved:

$$d = \frac{0.5 \lambda}{\mu \sin \alpha}$$

Since for visible light the smallest wavelength that can be used is in the neighborhood of 4000 Angstrom Units, and the maximum index of refraction of the fluid immersing the object is 1.7, the smallest distinguishable distance is about 1200 Å even if $\sin \alpha$ is given its maximum value of unity.

Figure 1. Arrangement of fundamental elements of (1a) light and (1b) electron microscopes. Figure 2. Image formation by scattering. Figure 3. Diagram of RCA Electron Microscope. Figure 4. Object chamber and air lock. Figure 5. Photographic chamber and air lock. Figure 6. Photograph of RCA electron microscope. Figure 7. Placing specimen on supporting film.



1a



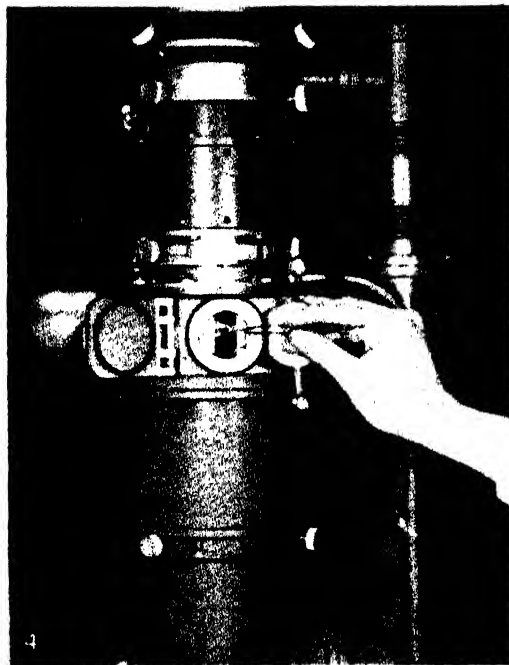
1b



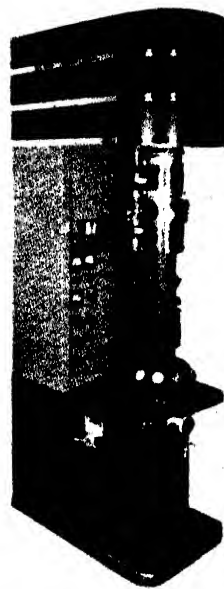
2



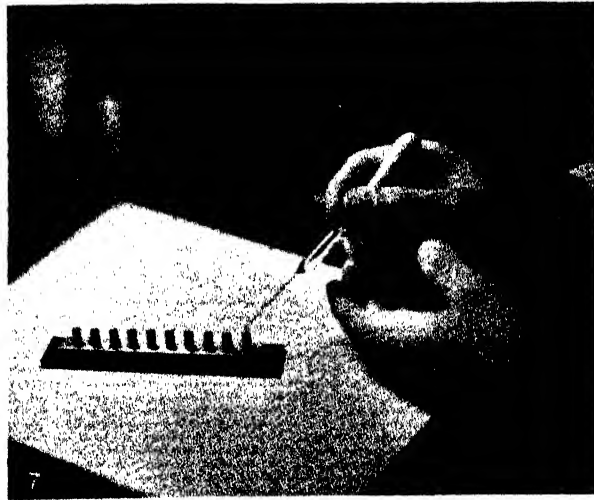
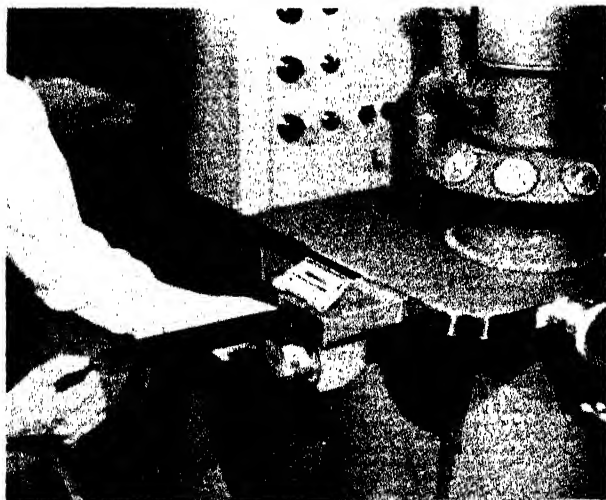
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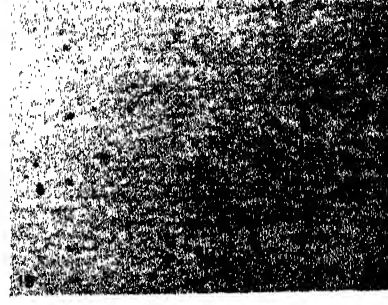
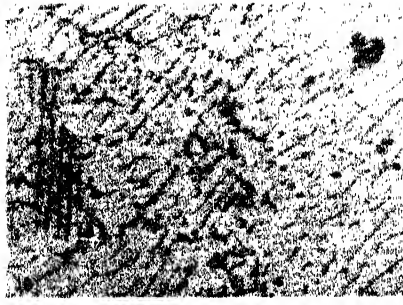
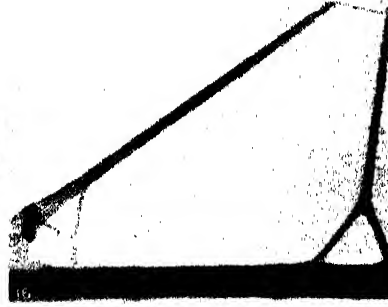
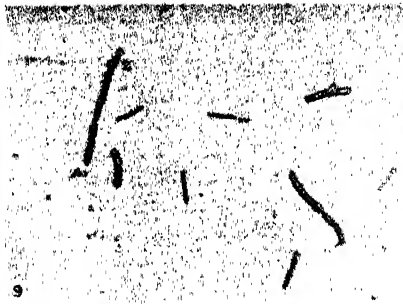
4



6



7



Where high-velocity electrons are used as the observing medium, the minimum distinguishable distance is very much smaller. The effective wavelength of electrons in motion is given by

$$\lambda = \sqrt{\frac{150}{V}} \text{ Angstrom Units}$$

where V is the electron velocity in volts, a relation which is derived from the wave mechanics of matter. Electron velocities of 60 kilovolts or more are used in practical electron microscopes, and hence the effective wavelength is less than 0.05 Å, or one-one hundred thousandth that of light. The angular aperture at the object is determined by the spherical aberration and the method of imaging. For objectives such as are used in present day electron microscopes, the aperture is of the order of one or two thousandths of a radian. Hence objects as small as 10 or 20 Å theoretically can be resolved. This is in close agreement with the observed performance of the modern electron microscope.

As mentioned above, the size of the objective aperture is related to the method of forming the image. In the conventional light microscope, light passing through the specimen is absorbed in varying amounts at different points; the variation in transmission producing the differences in intensity of light in the final image. In the electron microscope the specimens used are usually completely transparent so that all the electrons striking the object are transmitted through it. However, although electrons are not absorbed by the specimen they are scattered, the amount of scattering at each point being a function of the density and thickness of the object. This is illustrated in Figure 2. Electrons which are scattered through more than a certain predetermined angle are intercepted by the limiting aperture and therefore the electron density in the image will vary with the thickness and density of the object. It will be evident from this that the contrast depends upon the size of the limiting aperture and that, leaving aside all considerations of spherical aberration in the objective lens, a small aperture must be used if high contrast is to be obtained.

The electron projection lens, which forms the final image from the intermediate image, does not limit the attainable resolving power. The angular aperture of the electron ray bundles entering the projection lens is so small that spherical aberration effects in this element are entirely negligible.

Figure 3 shows the construction of the RCA electron microscope. The cathode and electron gun

are located at the very top of the instrument. Below are the condenser lens, the objective lens, and the projection lens. The specimen is held in the object chamber which will be described later. Finally there is a fluorescent screen pivoted in such a way that it can be moved into position to receive the electron image or rotated back so that photographic plates placed below it are exposed. The entire electron optical path is maintained at a pressure of about 10^{-5} mm of mercury by an oil diffusion pump.

The power supplies for the overall microscope voltage and for the electron lens coils are located in the cabinet which forms the rear portion of the instrument. These power supplies require very careful design, since the slightest variation in overall voltage or in lens current tends to defocus the instrument. Special circuits are provided which hold the high voltage and the objective lens current to a constancy of one part in fifty thousand. The condenser and projection lenses do not require quite as careful regulation, and are made constant to 0.02 and 0.004 percent respectively. With this degree of stability, the electrical circuits impose no limitations on the resolving power, even below 10 Å.

The volume of the main chamber of the present microscope is rather large, and if it were necessary to let air into the instrument and re-pump to high vacuum each time a specimen is changed, considerable delay would be caused. To avoid this an air lock is provided at the object chamber, arranged so that the specimen can be moved into a small chamber which can be sealed off from the rest of the instrument. Therefore, to remove the specimen it is necessary to let air only into this small compartment. The new object is then placed in the chamber, the chamber evacuated, and the object moved into its place above the objective; the whole operation of changing a specimen requiring only sixty seconds. The air lock and object chamber are illustrated in Figure 4. Delicate controls are provided to give transverse motion of the object in two directions, thus permitting the observer to view any desired portion of the specimen.

It is, of course, also necessary to change photographic plates during operation of the microscope. Therefore another air lock, shown in Figure 5, is provided for the photographic chamber. The photographic plates used with the microscope are long enough so that a number of exposures may be made on the same plate, and an adjustable mask allows the width of the picture to be controlled at will.

The complete microscope, shown in Figure 6,

Figure 8. Forming film by floating in water. Figure 9. Light microscope photograph of *Bacterium megatherium* ($\times 250$). Figure 10. Electron micrograph of *B. megatherium* ($\times 9,000$). Figure 11. Electron micrograph of *Aerobacter cloacae* ($\times 13,250$). Figure 12. Electron micrograph of human tuberculosis ($\times 8,000$). Figure 13. Electron micrograph of tobacco mosaic virus ($\times 12,500$). Figure 14. Electron micrograph of zinc oxide ($\times 11,000$). Figure 15. Electron micrograph of lead arsenate ($\times 15,000$). Figure 16. Electron micrograph of polymerized vinyl chloride ($\times 25,000$). Figure 17. Electron micrograph of slightly polymerized vinyl chloride ($\times 15,000$). Figure 18. Electron micrograph of pearlite (Modification of etched carbon steel) ($\times 6,250$). Figure 19. Electron micrograph of bainite (Modification of etched carbon steel) ($\times 5,750$).

stands about seven feet high and occupies not more than five square feet of floor space. It is completely shielded from electrical and magnetic disturbances. Thus it can be fitted into any research laboratory, and does not require a special shielded room to house it, as did earlier instruments.

Specimens to be examined in the electron microscope must be mounted quite differently from those observed in an ordinary microscope, since the electrons will not penetrate the usual glass slide. Figure 7 illustrates the procedure most frequently used for mounting objects for examination, which is to suspend them in pure water or other suitable liquid, then to place a drop of the suspension on an extremely thin cellulose film, which is supported on a fine mesh screen. The supporting film, which is of the order of 100 Å thick, is made by spreading a droplet of a solution of the celluloid on water. The formation of such a film is illustrated in Figure 8. Other procedures are to suspend the object particles on the cellulose itself, or, where the object is self-supporting, to mount the specimen directly on the wire mesh.

The performance can best be described with illustrations of electron micrographs made with the instrument. In the fields of biology and bacteriology the new microscope is a tool of immense importance. Figures 9 and 10 are photographs of *B. megatherium*—Figure 9 was made with a good light microscope, Figure 10 with the electron microscope. The former shows a resolving power of about 2000 Å, while objects less than 100 Å in size can be resolved on the latter. In Figure 11 are some specimens of *Aerobacter cloacae* showing the flagella by which the organisms attain mobility. Figure 12 illustrates the organism responsible for human tuberculosis. Filterable viruses, which have heretofore been unobservable because they are beyond the range of the optical microscope, can be easily resolved with the electron microscope. Tobacco mosaic virus is illustrated in Figure 13.

As great as is the importance of this instrument in biology and bacteriology, its value to research and industrial chemistry is fully as great. For instance details of surface conditions far below the resolving power of a light microscope have a considerable effect on absorption and other chemical properties. Figure 14 illustrates the appearance of a chemical preparation where the size of the particles is too small to be resolved with light. Another commercial chemical product having a plate-like character unrevealed with an optical microscope is shown in Figure 15. Figures 16 and 17 illustrate the difference between vinyl chloride which is fully polymerized and the same compound only slightly polymerized.

The applications of the microscope even extend into the realm of metallurgy, although this is an entirely new field and the techniques are not yet fully developed. Two electron micrographs of etched steel samples are shown in Figures 18 and 19.

These micrographs illustrate not only the wide

range of applications to which the electron microscope can be adapted, but also its tremendous resolving power. Some of the pictures show details as fine as 30 Å, in other words, between 50 and 100 times smaller than can be seen with the best optical instrument.

DISCUSSION

GATES: What is the length of the exposure you use?

ZWORYKIN: About 10 seconds. For observation a much greater intensity is used; for an exposure, the electron intensity is then reduced to about 1/50 and the picture is taken.

DELBRÜCK: How strong is the ionization dosage during the exposure?

ZWORYKIN: Very roughly about one micro-ampere per square millimeter on a film about 100 Å thick, and a maximum object thickness of about 1/2 micron.

FANKUCHEN: Is this for the actual picture or for viewing?

ZWORYKIN: This is for exposure; for viewing, about 50 microamperes per square millimeter is used.

FANKUCHEN: Can you take a picture without viewing?

ZWORYKIN: If you use the same specimen you can take repeated pictures of different fields of view without refocussing. When the specimen is changed it is necessary to refocus.

FANKUCHEN: I was thinking of biological specimens which could be damaged.

ZWORYKIN: If the intensity is not too high, you can notice no appreciable damage. If the intensity is too great, sometimes you break the supporting film or cause a change in the thicker parts of the specimen.

MULLER: This question has its economic side—what is the cost of the microscope?

ZWORYKIN: Those delivered already cost \$9500 FOB Camden, but now the price is slightly higher. There is no change in design.

MULLER: I thought there was reported to be a reduction owing to improved technique of manufacture.

ZWORYKIN: No.

MCINNES: What is the advantage of the higher voltage instruments?

ZWORYKIN: We find greatly increased penetration with higher voltage. Up to the present we have gone as high as 300,000 volts.

MULLER: Are the high voltage instruments for sale, and if so, what is their cost?

ZWORYKIN: They are not for sale. Their cost would be much greater, perhaps about \$50,000.

UBER: Do you realize a higher resolving power with the 300 kilovolt instrument?

ZWORYKIN: No.

UBER: The depth of the field is relatively very great; what is the explanation for this?

ZWORYKIN: The lens and illumination have a very minute aperture; the smaller the aperture, the greater the depth of focus.

DELBRÜCK: What determines absorption, nuclear scattering or ionization?

ZWORYKIN: Primarily ionization. However it is scattering that is for the most part responsible for intensity differences in electron images.

GATES: The fine structure of diatom frustules is still in question; have you photographed them?

ZWORYKIN: Yes; they show rows of openings but we did not make a detailed study.

BANTA: In the study of chromosome structures, long drawn out chromosomes should be more useful than salivary chromosomes. Is it probable that in the spireme a banded structure would be visible?

NEBEL: We photographed sections of leptotene in *Tradescantia* and found indications of banding. The test should be repeated.

MULLER: Very thin sections of rapidly frozen proteins might give "wet" rather than dry proteins to study. Is it not possible to take pictures of such "wet" specimens in the frozen state?

ZWORYKIN: You cannot use wet material in vacuum without protecting it in some way. With

small particles, like bacteria and blood cells, the cell membrane is so strong that water does not evaporate through it. If red blood cells are photographed wet, with low voltage they are opaque, with increased voltage they begin to be transparent but no structure is seen. Alternative explanations are: 1) they may have no structure, 2) Brownian movement may obscure it. We are trying to create a special cell containing liquid. The biggest trouble is loss of water through the collodion.

MULLER: Do you have means of keeping the specimen frozen while under observation, and if so, would the water sublime from it too quickly if not covered?

ZWORYKIN: We do have means of keeping the specimen cold but the wires of the grid might not conduct away the heat quickly enough.

BOCHE: Would not water if trapped scatter almost as much as protoplasm would?

ZWORYKIN: It would scatter and would decrease resolving power, but the resolving power is still better than that of the light microscope.

FANKUCHEN: Is it resolving power or contrast that is meant? Water is of about the same density as live material, so one could not see much detail.

ZWORYKIN: I mean resolving power, through the presence of water also decreases the contrast.

X-RAY DIFFRACTION STUDIES OF PROTEIN PREPARATIONS

I. FANKUCHEN

This is a symposium built around the central topic of genes and chromosomes. From the X-ray diffractionists's point of view one could up to now say very little about these most interesting subjects. The one direct X-ray experiment on chromosomes that has so far been performed gave disappointing results. I shall therefore compromise by saying nothing about chromosomes and genes and will instead discuss X-rays as used in the study of protein preparations.

The investigation of protein preparations by X-ray diffraction techniques started about twenty years ago. Astbury (1934) and Astbury and Bell (1938) have, in previous symposia here, given an adequate history and description of such investigations up to 1937, particularly about those concerned with the fibrous proteins. The techniques used in the study of crystalline proteins by Bernal and his school and those used in studying the plant virus proteins by Bernal and the writer, are however very different from those used in fiber studies and inasmuch as they are not generally known, merit a brief description.

The importance of the preparation of the specimens has not been sufficiently emphasized and as Astbury has pointed out many of the early failures to obtain either sharp diffraction patterns or useable data from what appeared to be perfectly good crystalline material were due not so much to inadequate X-ray technique as to improper specimen handling. Thus with crystalline proteins, if useful information is to be obtained, single crystals must be used, regardless of how small they may be. The powder technique is almost useless for all but questions of identification. Moreover, protein crystals immersed in their mother liquor give far better patterns than air dried crystals. Thus for horse methemoglobin (Bernal, Fankuchen and Perutz, 1938; Perutz, 1939) the wet crystals give patterns containing thousands of different reflections while air dried crystals with difficulty yield a meagre half dozen diffuse reflections. To handle small crystals under difficult conditions, special techniques have had to be developed, techniques which will be described in a subsequent paper (Bernal, Crowfoot, Fankuchen, Perutz and Riley, in preparation).

Just as is the case for crystalline proteins, the use of unorganized specimens for X-ray study of any complex material will usually give patterns which are not interpretable. In crystalline proteins, the order is obtainable, as we have seen, by the use of single crystals, but when the material is not obtainable as a crystal some other ordering is essential. Usually the elementary bodies or molecules are

physically anisotropic and then orientation by a variety of methods becomes possible. Naturally fibrous materials and materials which can be made into fibers usually contain anisotropic particles whose lengths are parallel to the fiber axis. This ordering is usually sufficient to permit some use to be made of the diffraction data. However the higher the degree of orientation or order imposed upon the specimen, the more complex generally do the patterns become and paradoxically enough the easier it is to make use of the data (because there are more of them). Thus Astbury (Astbury and Sisson, 1935) has been enabled by artificially imposing a secondary orientation upon protein fibers to demonstrate directly and conclusively that the "side chain" and "backbone" spacing were actually at right angles to one another, a conclusion which previously had been arrived at by rather indirect reasoning.

In the work on tobacco mosaic virus (Bawden, Pirie, Bernal and Fankuchen, 1936; Bernal and Fankuchen, 1937 and in press), the preparation of ordered specimens was essential to the X-ray work. Thus the first X-ray work (Wyckoff and Corey, 1936) on tobacco mosaic virus used disordered specimens and while very fine powder patterns were obtained, not much useful information could be derived from them.

When very small protein specimens are studied, both their size and the fact that they are large complex compounds and therefore scatter significantly at small angles impose certain restrictions on the apparatus used. The longer the wavelength of the radiation (Bragg's law is $\lambda = 2d/n \sin \theta$) used, the larger become the angles involved. However, practical considerations such as ease of production, absorption in air, etc., make it desirable to use the characteristic radiation of copper for most work. This choice makes it necessary to use very sharply defined beams of X-rays in order that the scattering close to the central beam may be studied. Also if small specimens are used, it is essential that the X-ray beam dimensions be made of the same order of magnitude as the specimen. Only then can the interfering air scattering (unless a vacuum camera is used) be kept down to a minimum and the weak but significant coherent scattering be observed.

When adequate precautions are taken, good useable diffraction patterns can be obtained from most crystalline proteins and from many non-crystalline but ordered biological systems. The interpretation of such patterns falls naturally into two steps, the study of the geometry of the patterns and the analy-

sis of the intensities of the individual reflections. The first step is quite straightforward and gives information about the geometry of the fundamental units (the unit cells) which, piling up regularly on one another, form the specimen. The second step is very involved and so far only in a few cases (Crowfoot, 1941) has such analysis been attempted.

For protein preparations which are not single crystals (fibers etc.) even the geometrical analysis may be difficult. Often the internal regularity and the degree of order are inadequate for a sure geometrical analysis. As the data become poorer, the possibility of alternative explanations for a given set of observations becomes greater and the fact that a geometrical picture does not disagree with the X-ray data is no longer good evidence for the essential correctness of the picture. It is then necessary to bring in evidence derived from other experimental techniques to test our ideas. But even then, the scientist should remain suspicious of his theories for an incorrect picture accepted on inadequate evidence is far worse than no picture at all.

There is an intermediate and not clearly describable stage between the two steps of the analysis which I feel is particularly important in the study of complex materials which even when crystalline are rarely very good crystals. One may describe it as a consideration of the "quality" of the diffraction phenomena. Whereas in the geometrical analysis and in the intensity analysis one deals with quantitative data, this intermediate stage has more to do with the qualitative aspects. There are no hard and fixed rules for this intermediate step; instead experience and even intuition are called upon. Thus we may observe that in protein patterns there is a general enhancement of the intensities corresponding to spacings of about 10 Å and 4.5 Å (Bernal, 1939); that reflections for crystals immersed in their mother liquor are sharper than those from dry crystals and extend out to much greater angles (corresponding to smaller spacings), that in wet crystals there appears to be a fairly sharp cut off at an angle corresponding to the water halo, and that certain reflections are sharp and others have tails or are otherwise smeared out. Such qualitative observations must have explanations in the deviations of the structures from the perfect regularity of ideal crystals and often we can guess at the explanations, guesses which may help in then carrying out the second and much more difficult stage, the analysis of the intensities.

X-ray studies of crystalline proteins have already contributed much to our knowledge of proteins. However, this work has been adequately reviewed several times in recent years (Crowfoot, 1941; Bernal, 1939; Fankuchen, 1941) and therefore the major part of this paper will be devoted to a summary of some X-ray studies which Bernal and the writer made of plant virus proteins first at the University of Cambridge and then at Birkbeck College, University of London. A detailed account of

this work is now in press (Bernal and Fankuchen, in press). There are two reasons for describing this work here—we found the plant viruses most interesting to study and I am assured by biologist friends that there is possibly a close relationship between viruses and genes.

Dr. Stanley will no doubt tell you about the isolation and chemistry of the viruses we worked with and so I shall confine my remarks to the X-ray work itself.

The initial optic studies and other physical properties of virus solutions indicated clearly the presence of very long particles and consequently it was possible to prepare by various means, specimens of differing concentrations in which at least one degree of order was present, namely the particles were all parallel to one another. Preparations of 50 percent or more protein were gel-like and are referred to as wet or dry gel depending on the water content. Solutions down to about 2-5 percent protein (depending on purity) are spontaneously birefringent and when introduced into fine capillary tubes, became ordered due to the particles lining up parallel to the capillary length. More dilute solutions which were isotropic when stationary were ordered by flow through capillary tubes.

The first X-ray studies were of dry gels and used very simple apparatus similar to that used by Astbury for his studies of textile fibers. The patterns obtained were somewhat like his fiber patterns with one important difference. If the specimen is considered as oriented so that the direction of particle length is vertical, then on the photographs a very marked low angle horizontal (equatorial) scattering was observed. When apparatus using finer X-ray beams and much longer specimen to film distances was used to study this low angle scattering, it became resolved into a pattern of sharp lines (Bawden, Pirie, Bernal and Fankuchen, 1936). When specimens of varying protein concentration were studied, it was observed that the large angle scattering did not change appreciably whereas the low angle pattern of equatorial reflections shifted regularly with the protein concentration, moving in to lower angles as the protein concentration decreased. The interpretation is fairly obvious; there is a dual pattern present, the wide angle unchanging pattern can only have its origin in regularities existing within the virus particles while the low angle scattering must be due to regularities in the arrangement of the particles relative to one another, an arrangement whose metrical relations change with the protein concentration. The X-ray work thus naturally falls into two classes, the study of the low angle or intermolecular patterns and of the wide angle or intramolecular patterns. The intermolecular results will first be presented.

INTERMOLECULAR STUDIES

a) *Non equilibrium gels.* If a small piece of oriented dry gel is wet slightly, it absorbs the water

and expands. Further addition of water results in further expansion and the complete immersion of the gel in water will result in its eventual disintegration. Preparations such as dry gel, wet gel and solutions of virus protein were termed non-equilibrium gels as the particles distributed themselves as widely as was possible. The dry gel pattern (see fig. 1a) consisted of at least 6 lines, the first four of which were most intense. The spacings corresponding to the lines varied inversely as $\sqrt{1}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{7}$, $\sqrt{9}$, etc. Now these ratios are exactly those which one would expect from an hexagonal arrangement in cross section of a bundle of parallel particles and consequently such an arrangement was taken as representing the structure of dry gel. (The arrangement just falls short of being a three dimensional crystal because the ends of the particles bear no regular arrangement relative to one another.) As the intermolecular patterns of the series of non equilibrium gels were obtained, one could see a steady increase of the spacings as the water content increased (fig. 1b, 1c). The patterns became progressively worse and the number of observable lines also diminished. However, their ratios were always those corresponding to an hexagonal packing. Furthermore, the interparticle separation increased inversely as the square root of the concentration the relation being $I. P. D = 1650/\sqrt{N}$ where N is the number of grams vacuum dried virus in 100 cc. of specimen. Such a relationship would hold only if the separation were only in a direction normal to the particle length. Here then is direct evidence that the virus particles can maintain a parallel regular arrangement *even when they are separated by several hundred Angstroms of water*. The arrangement moreover is a very perfect one. In Figure 1c the bottoms of the pair of lines possess sharp tails which are asymmetric doublets. Crystal monochromatized radiation was used for this experiment and the slit system permitted the passage of the $K\alpha$ doublet. The hexagonal arrangement was thus perfect enough to maintain the resolution of this very close doublet. Figure 1d is another solution in which the sharpness of the reflection indicates the perfection of the specimen for the entire length of the slit system. The intramolecular pattern given by the well arranged part of Figure 1c is shown in Figure 2. The sharpness of the reflections here indicates that the particles which Fig. 1c indicated formed a very exact hexagonal arrangement in cross section are also parallel to one another within a degree or so—all this in a preparation in which the particles are separated by their own diameter, 150 Å.U. of water.

b) *Equilibrium gels*. If a piece of dry gel is immersed in buffer solutions or in salt solutions then there is a range of pH values and ionic concentrations within which the gel will take on a limited amount of water and swell to an equilibrium value. A series of intermolecular patterns indicates that the particles in such preparations stay parallel

to one another and that the cross sectional arrangement continues regular and hexagonal. In the buffer solutions, equilibrium gels exist between pH values of 3.0-7.0 with a minimum interparticle separation of about 180 Å.U. at pH 3.4 the isoelectric point.

In solution of ammonium sulphate, dispersion occurs for concentrations less than one fourth normal but equilibrium occurs at higher concentrations, the interparticle separation falls steadily with increasing ionic concentration from a maximum value of about 325 Å.U. to a minimum of about 170 Å.U. for solutions of four or higher normality. These results are valuable in indicating qualitatively the role that electrical forces play in colloid structures but what is more important furnish directly a quantitative parameter, the interparticle distance, which should be very useful in the testing of theories of colloid structure. Certainly the study of intermolecular patterns of both equilibrium and non-equilibrium gels reveals the existence of powerful electrical forces in protein preparations. It still remains for the theoretician to give them a quantitative explanation.

Obviously such studies as are here reported are of a very preliminary nature. Similar investigations of the effect of different reagents such as alcohol, acetone, aqueous solutions of different salts, the effect of temperature upon the equilibrium conditions would undoubtedly help much in achieving a better understanding of the electrical forces and therefore of the particles (or molecules?) wherein they have their origin.

INTRAMOLECULAR STUDIES

I do not wish to go into a detailed description of the intramolecular pattern. For such a detailed study, one is referred to our paper in the JOURNAL OF GENERAL PHYSIOLOGY (in press). However there are certain features which may appropriately be discussed here. Figure 2 is the best intramolecular pattern so far obtained. It is worth emphasizing that it is so good only because the specimen was perfectly ordered and regular. It is the pattern of a 36 percent solution of enation strain of tobacco mosaic virus and is far superior in detail to the intramolecular pattern obtained from the more concentrated dry gels. This is not so surprising as it may appear because in the solutions the possibility exists of the particles lining up very parallel to one another while the very process of drying which is necessary to make dry gels produces cracking and other dislocations which affect the ordering of the specimen. While this particular pattern shows more detail, yet a comparison of the intramolecular patterns of oriented tobacco mosaic virus preparations of varying concentrations reveals no significant changes. We may conclude that this pattern does arise in the periodic structure of the particle itself, a structure which is in a sense a small three dimensional crystal and that, moreover, the particle is comparatively unchanged in preparations rang-

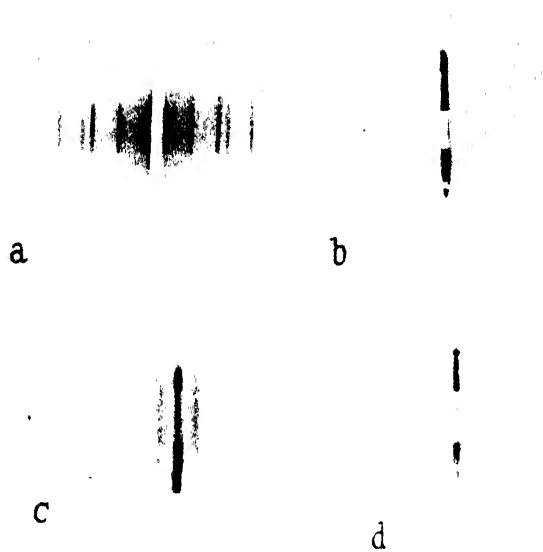


FIG. 1. Intermolecular patterns of virus preparations of varying concentration. Copper $K\alpha$ radiation $\lambda = 1.54 \text{ \AA}$. U.

Condition of specimen		Specimen to film distance
a dry gel	ordinary strain	40 cm.
b wet gel	ordinary strain	30 cm.
c 36% solution	enation strain	40 cm.
d 18% solution	ordinary strain	40 cm.

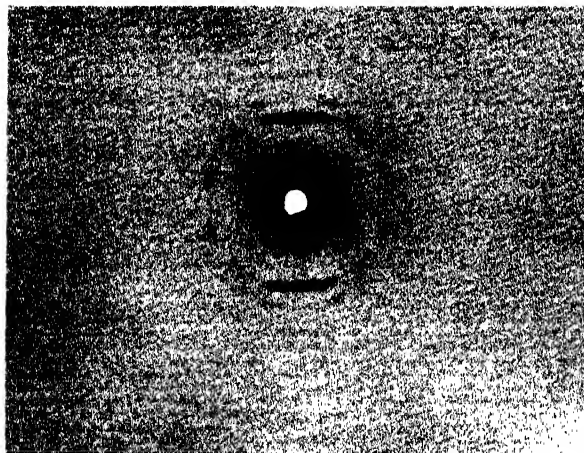


FIG. 2. Intramolecular pattern of enation strain of tobacco mosaic virus protein. Specimen to film distance, 8 cm ; radiation used, copper $K\alpha$ $\lambda = 1.54 \text{ \AA}$. U.; concentration of protein, 36%; time, 170 hours.

ing from the vacuum dried gel to 2 percent solutions. This observation may be important in formulating our ideas of the structure of proteins particularly if this relative constancy of the molecule is found to extend beyond the limited virus proteins here described.

If an unoriented specimen had been studied using the same technique, then the result would have been a series of sharp rings, as each spot in Figure 2 would have been smeared out into a ring of radius equal to the spot's distance from the center of the photograph. The first X-ray studies (Wyckoff and Corey, 1936) were indeed so made and the pattern was a series of rings. It is interesting that in this case an erroneous conclusion was arrived at which was almost inescapable at the time because the specimen was not ordered. It was entirely reasonable to consider a sharp line pattern as evidence for the crystalline character of the material and the conclusion was so drawn but in the light of the studies of the intramolecular and intermolecular patterns of ordered specimens we see that the sharp ring system had its origin not in a crystalline arrangement of the particles relative to one another (which after all is not three dimensional crystalline) but rather in the crystalline character of the molecules themselves. This points clearly to the care which should be exercised in making use of the data from powder photographs of unoriented specimens of complex biological materials in which the possibility exists of what may be called superstructures.

The intramolecular pattern (fig. 2) contains much more information than unfortunately we now are in a position to use. The separation of the layer lines corresponds to a true periodicity along the molecule axis of 68 Å.U. while the obvious enhancement in the intensities of the third and sixth layers suggest pseudo sub units of 22 Å.U. and 11 Å.U. height. The distribution of spots along the layer lines can be interpreted in terms of an hexagonal unit cell of side 87 Å.U. but again intensification of reflections suggests pseudo periodicities of 44 Å.U. and 11 Å.U. The ring of spots of approximately 11 Å.U. spacing is indeed very marked in the pattern. There is some evidence from the intramolecular pattern as to the length of the particles. No exact value can be given but the sharpness of the reflections in the direction of particle length sets as a lower limit a length of at least twenty repeat units, roughly 1500 Å.U.

Some work has been done on other viruses. Cucumber 3 and 4 give intramolecular patterns very similar to those obtained from the various strains of tobacco mosaic virus but the intermolecular patterns indicate that the diameter of the cucumber virus particles is about 4 percent less than that of the tobacco mosaic virus strains, 146 Å.U. as compared to 152 Å.U. for the latter.

To summarize—X-ray studies of plant virus proteins have demonstrated the existence of elongated protein particles at least 1500 Å.U. long and 150

Å.U. in diameter. The electrical charges on the particles are the source of forces capable of effects over hundreds of Å.U. of solution. The particles themselves have a regular internal structure capable of producing three dimensional X-ray diffraction effects. This pattern has been partly interpreted in terms of a particle structure which is hexagonal $a = 87$ Å, $c = 68$ Å. The disposition of the X-ray intensities further suggest smaller pseudo units, the smallest of which is approximately equidimensional of side 11 Å.U.

X-ray work on proteins have given much information about their structure and physical properties, particularly their sizes, shapes and electrical properties as manifested by the way they pack. In so far as chromosomes and genes are largely protein, an increased knowledge of proteins must lead to a better understanding of chromosomes and genes, of their structure and what is equally important their behavior.

Estimates of the sizes of genes give values of the same order of magnitude as those of the virus particles. Despite the failure so far to obtain useful X-ray data directly from chromosomes, the situation does not appear completely hopeless. The largest chromosomes can be measured in fractions of a millimeter. The writer has obtained single crystal oscillation photographs of protein crystals $.01 \times .01 \times .1$ mm. in size and recently by the use of micro X-ray methods diffraction patterns of single ramie fibers. $.01$ mm. in diameter. The possibility really exists of studying by X-ray diffraction methods single chromosomes. Whether the data when it is obtained will prove useful is something which cannot be prejudged now; but we can say that single chromosomes are not too small to be studied by this technique.

It is important to realize that this virus work has shown that X-rays can be used to determine not only interatomic arrangements (what may now be called classical X-ray diffraction work) but that the low angle scattering can be used to study the size, shape and distribution of larger scale structures which may contain millions of atoms. Low angle studies may be able to differentiate between Painter's bundle of thread concept of the salivary gland chromosomes and the alveolar structure of Metz and it may even tell us something about the size and distribution of genes along the chromonemata.

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DISCUSSION

WRINCH: What is the maximum distance over which the forces are observed?

FANKUCHEN: As large as 500 Å. The farther apart the particles go, the less protein present in any given volume, and therefore with decreasing protein concentration we find longer exposures are needed. Even for the most dilute solutions for which intramolecular patterns were obtained, the angular distribution over which the reflections extended indicated very good parallelism of the particles. Since intermolecular patterns could not be obtained for such dilute solutions, one cannot say whether the arrangement was still hexagonal or not, but there is no good reason for suspecting it was not.

CHILD: What are the geometry and dimensions of the repeat units?

FANKUCHEN: The layer line separation gives definite evidence as to the periodicities along the particle axis. The primary repeat is 68 Å units but the intensification of the third and sixth layer line spots indicates the presence of sub-periodicities of 22 and 11 Å. The cell dimensions at right angles to the molecular axis at first suggested an hexagonal unit cell of 88 Å side. Some anomalous reflections were observed which did not fit this cell. These could at first only be explained by a cell larger than the particle itself, an impossible situation which was resolved when it was realized that they were probably secondary maxima arising from the small number of repeat units in the cross section. Various subunits are also suggested by the intensification of various reflections, a ring of spots at about 11 Å suggesting the size of the smallest subunit as about 11 Å cube.

DEMEREK: How does this fit in to a diameter of 150?

FANKUCHEN: Three hexagonal unit cells of side 88 Å units would fit in very nicely to a particle of diameter 150 Å.

BOCHE: Several years ago, Astbury, Metz, Buck, Melland and I tried to get diffraction photographs of *Chironomus* salivary gland chromosomes without success. Astbury, who took the X-ray photographs, reported that no appreciable orientation was detectable. Maxwell then tried electron diffraction, using 40 KV, which was not enough to get through the material; the chromosomes were apparently too thick. Recently we used the RCA diffraction camera operating at 250 KV and got no evi-

dence of orientation either although the material was frozen and dried.

FANKUCHEN: All these experiments studied only scattering at angles corresponding to spacings smaller than about 10 Å. There is no *a priori* reason why one should expect the individual polypeptide chains, whose structure and disposition relative to one another would be responsible for such spacing, to have specific orientation. Rather if orientation is present in chromosomes it is the orientation of protein organized on a larger scale, say threads of almost microscopic size. Consequently the X-ray evidence for such orientation can only be found by the study of scattering at small angles.

Sometimes dry material is good, sometimes it is bad. But some proteins give good patterns when dry; for example, ribonuclease obtained from Kunitz gives good patterns in dry crystalline preparations as well as in wet. Drying however always decreases the crystal perfection.

FANKUCHEN: This is similar to the behavior of virus aggregates.

SCHULTZ: Supposing there is a difference in pattern of long range forces between strains of virus, what would you get if the strains were mixed?

FANKUCHEN: I don't know. There may be the same kind of line pattern but I cannot say anything about the intensity of the lines.

SCHULTZ: This bears on the "legality" of invoking electrical forces to explain the attractive forces of genes.

STANLEY: Concerning the results from intermolecular patterns, I might mention that Bernal has related the long range force idea to chromosome orientation and the geneticists at the Stanford symposium on the cell and protoplasm seemed to like it. Previous to the work with tobacco mosaic virus there had been no evidence for these long range forces but now good theoretical grounds are known for their existence. Their existence is therefore supported by both theory and fact.

MULLER: Bernal's interpretation involved, so far as I know, no specific forces but only van der Waals forces, according to which synopsis between homologous parts would not be very much stronger than between non-homologous. Geneticists are inclined to infer that much more specific forces must exist. It seems to me that possibly this is of the nature of a resonance phenomenon on a bigger scale than that envisaged by the chemist. There is some biological evidence for the idea that synaptic forces may be of that type. It seemed to me, for example, the most plausible interpretation of inverted synopsis, as found by myself and Raffel in 1935, in the heterozygous scute¹⁰ inversion; here the two minute homologous parts of the chromosome which lie nearly opposite each other undergo synopsis even though they are inverted with respect to one another, a result which indicates that not spatial but vibrational agreement determines the attraction. Independently of this unpublished work, Jordan, a

few years ago, suggested a type of force in explanation of synapsis which I believe is of this type; Delbrück, however, has published objections to this interpretation. Nevertheless it seems to me that some account should be taken of the large scale nature of the forces which seem to be concerned in synapsis.

FANKUCHEN: Resonance forces are probably not adequate to explain this.

MULLER: Resonance forces are needed which would give the molecule some sort of supervibration.

STANLEY: Bernal thinks the forces may be exerted over a great distance, say 1500 Å.

MULLER: Did he have an explanation of the sources?

FANKUCHEN: You have charged regions on the particles, but the net forces probably do not have a specific character.

SCHULTZ: Bernal proposes that spindle growth is analogous to the growth of tactoids, and has not as far as I am aware related the long range forces to synapsis. By using these long range forces we can get a mechanism for spindle growth in the cell.

Another idea of Bernal's does concern synapsis: he suggested some time ago that from considerations of energetics, homologous structures would come together, the probability of like units coming together being greater than that for the unlike ones.

MULLER: But the probability is so weak that non-homologous synapsis would then be too frequent.

WRINCH: With regard to Schultz's first remark, I feel here that we have the possibility of a crucial experiment which may be of great importance in determining the direction in which cytogeneticists may most usefully look for a clue to the process of homologous attractions. The issue is fairly simple, namely, what if any difference would there be between X-ray pictures of two different strains and that obtained from a solution containing various proportions of two such strains. If there is a definite difference, this gives a possibility of some specificity in the forces we are now postulating. It should be emphasized that the essential need for the cytologist probably is for forces of highest possible specificity.

STRUCTURE AND MECHANICAL BEHAVIOR OF HIGHPOLYMERS

H. MARK

All our present experimental knowledge points in the direction that the mechanical behavior of Organic Highpolymers, such as proteins, cellulose, rubber, synthetic resins etc., is of a very complicated character. All these materials show a certain amount of reversible elasticity, the modulus of which varies between 10^6 and 10^{11} dynes per sq. cm., but they also exhibit flow, which leads to a permanent final elongation of the samples after stretching. The viscosities corresponding to this flow range between 10^8 and 10^{13} poises.

The problem is to correlate these mechanical properties with the molecular structure of these materials, i.e., the presence of long chains, exerting certain mutual van der Waals forces, having a certain average length and a certain internal flexibility. It has not yet been possible to work out such a correlation to the last detail and to predict quantitatively the total mechanical behavior of the material over a wide range of experimental conditions, such as magnitude of stress or shear, temperature, time of the experiment etc. But it has been possible to explain qualitatively the elastic and plastic properties over a certain range and to make a few quantitative predictions, which agree fairly well with the experimental facts.

Before describing the general situation in this field, it seems appropriate, first to enumerate the different types of elementary processes of elastic behavior and of flow, and then to attempt to build up the mechanical behavior of a highpolymer.

DIFFERENT TYPES OF ELASTICITY

There are several different elementary mechanisms, which involve complete reversible elasticity.

a) *Elasticity due to covalent forces:* If one stretches a single crystal of diamond, quartz or tungsten, one observes elastic behavior over a short range of elongation. The extension is proportional to the stress (Hooke's Law) and disappears instantaneously after the stress is released. The modulus of elasticity ranges between 10^{11} and 10^{12} dynes per sq. cm. ($10^5 - 10^6$ kg. per sq. cm. or $1.5 \times 10^6 - 1.5 \times 10^7$ lbs. per sq. in.) and the range of elastic extensibility is between 0.1 and 1.0 percent. It is generally assumed that the mechanism of this type of elasticity is the following: each atom or ion of the crystal vibrates around an equilibrium position, which is prescribed by the forces of all the other atoms or ions and can be described by a potential curve. The minima of this potential function are the rest positions of the atoms or ions, their distances determine the dimensions of the undistorted crystal lattice.

If one stretches such a system, each lattice point is lifted into a slightly higher level, and the equilibrium positions of the distorted lattice are now separated by slightly longer distances: the crystal has been extended and potential energy has been stored in it. This state can be maintained for an indefinitely long time, without any further change in the arrangement of the lattice points (except of fast vibrations around the equilibrium positions) because the energy barriers between the rest positions are comparatively high (50,000-100,000 cal. per mol.) and prevent (at least at room temperature) any movement of the particles in a certain direction under the influence of the stress (which would represent a flow).

Our present knowledge of the nature of the covalent bond as particularly developed and described in Linus Pauling's excellent book on the Nature of the Chemical Bond allows certain quantitative calculations and leads to the correct order of magnitude of the modulus of elasticity, of the range of the elastic extensibility and of the time of relaxation. As a rough approximation one can say: the depth of the potential holes accounts for the high time of relaxation ($\lambda > 10^6$ sec), while the shape in the immediate neighborhood of the minimum (radius of curvature) determines the modulus of elasticity and the range of Hooke's Law.

This type of elastic behavior is characterized by a high modulus ($E \sim 10^{12}$ dynes per sq. cm.), by a short range ($E\ell \sim 0.1\%$) and a high time of relaxation ($\lambda > 10^6$ sec.).

b) *Elasticity due to secondary valence (van der Waals) forces:* If one stretches a single crystal of cane sugar, phthalic anhydride or pentaerythritol one also observes elastic behavior over a short range of elongation. Again the Law of Hooke is fulfilled over a limit of 0.1 to 1.0 percent exertion, but the moduli of elasticity are now distinctly lower. They amount to $10^9 - 10^{10}$ dynes per sq. cm. At sufficiently low temperatures (100° C. and below), the time of relaxation is again high ($\lambda > 10^6$ sec.) so that now flow occurs and the sample assumes completely its original shape as soon as the stress is released.

The mechanism of this type of elasticity is presumably the following: Each molecule of the crystal is situated in the hole of a van der Waals potential curve and vibrates around its equilibrium position. The distances between these minima of the potential function are given by the intermolecular forces; they determine the dimensions and symmetry of the undistorted lattice.

If one stretches such a system, each molecule is

slightly lifted and the equilibrium positions of the distorted lattice are now a little bit further distant than before. The crystal has been extended in the direction of the stress and potential energy has been stored in it.

At sufficiently low temperatures this state can be again maintained for an indefinitely long time, because the energy barriers between the equilibrium positions prevent the transition of a molecule from one minimum into the next under the influence of the stress. However these barriers are now much lower than they are in the case of a covalent lattice, and such secondary valence lattices are inclined to flow at temperatures which are much lower than the corresponding temperatures of covalent lattices. Cane sugar, for example, has at normal temperatures still completely reversible elasticity ($E \sim 10^{10}$ dynes per sq. cm.; $E_l \sim 0.5\%$; $\lambda > 10^6$ sec.), while lauric acid exhibits already distinct flow ($\lambda \sim 10$ sec.). This is due to the fact that the secondary valencies between the OH-groups produce rather deep minima in the potential function, while the forces between the CH_2 -groups and the few COOH -groups in lauric acid produce only shallow and flat minima.

The quantitative treatment of intermolecular forces was mainly developed by Debye (1920, 1921), Keesom (1921, 1922), London and Eisenschitz (1930), Slater and Kirkwood (1931); it leads to energy barriers between 2000 and 10,000 cal. per mol. and allows a fair understanding of this type of elastic behavior. It can be characterized as a short range ($E_l \sim 0.1\text{--}1.0\%$) elasticity of a moderate modulus ($E \sim 10^9 - 10^{11}$ dynes per sq. cm.) with a high time of relaxation at sufficiently low temperatures. However, here, the critical range of temperature, at which flow sets in is in the neighborhood of room temperature. This makes it difficult to observe this type of reversible elasticity without the interference of flow.

c) *Elasticity due to secondary valence forces, if long chain molecules are present.* Mack (1932, 1937) has first drawn attention to the fact that van der Waals forces may produce a long range elasticity, if they act between the different segments of a flexible long chain molecule. Let us consider a chain of 1500 isoprene molecules (as present in rubber) or of about 1000 amino acid residues (as present in wool), which is folded together in such a way that the free mobility around the different bonds is used to bring certain segments of the chain into the sphere of their mutual van der Waals attraction. Owing to the fact that certain valence angles have to be maintained during this folding operation and due to the additional fact, that there is no complete free rotation about any single bond, it will not be possible to fold the chain together so tightly that all the van der Waals attraction centers can be completely saturated. A certain number of them will get into a position of complete saturation, another number will get nearly into such a position, while the rest will

remain free and therefore will not contribute to contraction energy (or internal van der Waals potential) of the curled up chain. It can be seen that this internal potential shall depend upon the number of attraction centers along the unit length (e.g. 100 Å) of the chain and upon their individual strength. It depends also upon the internal flexibility of the chain and upon whether these attraction centres are regularly distributed along the chain (as in rubber, proteins or nylon) or whether they follow each other in a more irregular and at random way (as in copolymers like buna N, buna S, Chemigum, Ameripol or Hycar).

If one stretches such a folded chain, one lifts the different segments into higher energy levels of their potential holes and stores energy in the system. Owing to the fact that not all van der Waals attraction centers contribute to this energy storing, the modulus will be lower than it was before. Taking into account the average van der Waals attraction potential of groups such as OH, COOH , CONH , CH_3 and considering the average flexibility and ability to fold of hydrocarbon and protein chains, one gets for this type of Mack-elasticity a modulus in the range between 10^7 and 10^9 dynes per sq. cm. On the other hand one has to expect that this elastic behavior will not obey Hooke's Law and will hold over a longer range of elongation. Due to the randomness in the positions of the different segments of the folded chain, some secondary valence bonds will be already stretched to their limit and eventually even opened, while others are still unstretched or only slightly stretched. This has the consequence that the modulus will gradually decrease with increasing elongation and that the amount of elastic extension will be comparatively high. If one takes chains of a polymerization degree around 1000, one may have reversible elasticity up to an elongation of 100 percent and more, while the modulus decreases as the chains get more and more unfolded and straightened out.

Altogether the Mack-elasticity is characterized by a low modulus ($10^7 - 10^9$ dynes per sq. cm.) a comparatively long elastic range (100% elongation and more, but not following Hooke's Law) range and by a high time of relaxation ($\lambda > 10^6$), if the temperature is sufficiently low to prevent any kind of irreversible flow.

d) *Elasticity due to the kinetic motion of long chain molecules.* If one has a number of long chain molecules (polymerization degree around 1000) with a certain degree of internal flexibility and considers their most probable arrangement, one finds that it does not correspond to the extended state of these chains, but rather to a state of a certain degree of curling up or folding together of each single chain. If one extends such a system one changes its entropy rather than its energy by decreasing the randomness in the arrangement of the chains. Hence the extended state of the sample corresponds to a lower entropy value and the system will show a

certain tendency to return into its original state of complete randomness (highest probability, largest value of entropy, lowest value of free energy). This tendency is not due to any specific forces between any individual parts or segments of the chains, but rather to the irregular, kinetic movement of their segments which results from the finite thermal energy content of the system.

Woehlisch (1927, 1928) first drew attention to this fact and Meyer (1932), Guth (1934, 1937), Kuhn (1934, 1936, 1939), Huggins (1938, 1939) and others have developed the quantitative treatment of this type of elasticity. It turns out that the modulus is of the order of magnitude between 10^6 and 10^7 dynes per sq. cm. and remains constant up to an extension of about 200 percent. This would correspond to a long range Hooke's Law elasticity with a low modulus. The kinetic elasticity as such would even obey Hooke's Law over a longer range, if not, crystallization would interfere as soon as the chains have been straightened to a certain extent (1941).

Altogether the kinetic elasticity is characterized by a low modulus ($10^6 - 10^7$ dynes per sq. cm.) by a long range of reversible proportional extensibility (200% and above) and by a high time of relaxation if the temperature is sufficiently low ($\lambda > 10^6$ sec.).

However it must be pointed out that the thermoelastic behavior of the kinetic elasticity is completely different from the cases a), b) and c). In these former cases the tendency of the sample to return into its original shape is due to the action of forces (interatomic, interionic or intermolecular), and increase of temperature decreases this tendency; the modulus of elasticity therefore decreases with rising temperature. In case d) on the other hand the contraction of the extended sample is due to kinetic rather than to potential energy and therefore the modulus of elasticity increases proportionally to the absolute temperature.

This short discussion shows that one has two distinct elementary mechanisms of elasticity as long as one confines the interest to low molecular weight compounds, but that one has to take into account four such mechanisms as soon as one wants to include the behavior of highpolymers. Particularly it can be seen that long range elasticity (above 100% extension) is closely connected with the presence of long chain molecules exhibiting a certain internal flexibility of the chains.

In this connection a problem might be discussed, which Dr. Shedlowsky kindly brought to the attention of the writer, namely, the question of the existence of long range forces between protein molecules in solution. If we assume that two particles having each a molecular weight of 5×10^6 (which corresponds to a diameter of about 200 Å) are at a distance of 2000 Å (2×10^{-5} cm.) from each other, then they are certainly outside the range of their mutual molecular attraction, even if one would assume very strong dipoles (or even ions) being dis-

tributed all over the surface of the particles. The distance between the two particles which amounts to about 10 times their diameters would also exclude any appreciable mutual interaction due to the disturbance of the flow of the surrounding solvent. We will now assume that these particles are connected with each other by 20 rubber chains, each of which has a length of 2000 Å in the completely extended state. Such a chain has to be built up by about 500 isoprene residues, which is about the order of magnitude of the average polymerization degree of commercial rubber samples. Twenty such chains would have a molecular weight of about half a million and would therefore amount to five percent of the weight of the two particles. These connecting chains would, according to the kinetic considerations mentioned under section d), represent a force of 10^{-7} dynes between the two particles. If they are completely extended they would approach the particles to each other, which would have to be interpreted as a force acting between them. In water (viscosity equal 10^{-2}) at room temperature the particles would assume the velocity of about 0.1 cm. per second, which means that they would move very distinctly under the influence of the connecting rubber chains. The force would, of course, decrease as the particles approach each other and would cease to act if their distance were around 50 Å. It seems therefore that rather small amounts (small by weight compared with the total system) of flexible chains which connect spherical particles may produce forces which have a long range of action.

A similar effect would occur, if two particles (which are not connected) having long flexible chains as feelers approach each other by Brownian movement, until the feelers touch each other and get connected by some type of condensation reaction (ionic or atomic forces). If the two feelers grow into one chain, the particles would attract each other due to the tendency of the chain to curl up. Each connecting chain would represent a force of about 5×10^{-9} dynes as long as the chain is completely or nearly completely stretched. The cases a, b, c, and d represent the most important individual mechanisms of elasticity in a condensed system, such as a crystal or a long chain compound. They are the elementary processes, by which one will have to build up the behavior of more complicated systems such as highpolymers, which are partly crystallized and partly amorphous, glasses or swollen gels.

However as all those substances exhibit also a certain amount of flow it may be appropriate to discuss shortly the elementary processes of flow, before passing over to the highpolymers.

THE ELEMENTARY PROCESS OF FLOW

If a shear τ produces a velocity gradient dv/dx , which is proportional to τ , one calls the system under consideration a true liquid, which obeys Newton's Law:

$$\frac{dv}{dx} = \varphi \cdot \tau = \frac{1}{\eta} \cdot \tau$$

According to Bingham φ is called the fluidity and η the viscosity of the liquid.

Several molecular theories have been advanced to predict the viscosity of a liquid from the size and shape of its molecules and the forces between them. van der Waals (1918), Andrade (1934) and Herzog and Kudar (1933) have developed formulae, which succeed in agreeing fairly with the observed values under different experimental conditions, such as pressure, temperature, etc. The most general and successful theory of rate processes in liquid systems was recently worked out by Eyring and his collaborators (1936, 1937). In the sense of this theory the fluidity of a system is a measure for the probability of a molecule moving under the combined influence of the thermal movement and the shear, from the place which it occupies into another place, such that this elementary jump of the molecule attributes to the flow of the liquid. It can be shown that the average time between two such movements of a molecule is proportional to the viscosity. As much longer as it takes until a particle can carry out such an elementary step of flow, so much slower will be the flow at a given shear and consequently so much higher the viscosity of the system.

Thus from the point of view of the molecules, viscosity measures a time (the time of relaxation), namely, the time which has to elapse until the molecule succeeds in moving under the influence of the stress into another position. This time is of course inversely proportional to the probability for such a step. The probability is determined by the volume of the particle which has to move and by the activation energy, which it has to accumulate until it can superate the energy barrier, which holds it on its place.

The viscosity of water (10^{-2} poises) corresponds to a time of relaxation $\lambda \sim 10^{-12}$ seconds, which means that a water molecule, if put under a certain stress, succeeds very quickly (in 10^{-12} seconds) in releasing this stress by moving into another (unstrained or less strained) position. All normal solvents, such as benzene, water, acetone, butyl alcohol, etc., have viscosities between 10^{-3} and 10^{-1} poises, corresponding to times of relaxation of 10^{-13} to 10^{-11} seconds.

Lubricants, such as mineral and vegetable oils are in the range between 10^{-1} and 10^1 poises, or 10^{-11} and 10^{-9} seconds. Spinning—and casting—solutions (cellulose acetate, cellulose xanthate, etc.) have viscosities from 10^1 to 10^3 poises and λ -values from 10^{-9} to 10^{-7} . Molding and glass blowing viscosities range from 10^3 to 10^6 poises (λ from 10^{-7} to 10^{-4}). The viscosities of swollen gels, such as swollen viscose rayon, gelatine, rubber, wool, cellulose nitrate and acetate range from 10^6 to 10^{10} poises (λ from 10^{-4} to 10^0), while the air dry samples have

viscosities from 10^{10} to 10^{14} (λ from 1 to 10^4 seconds).

In the case of highpolymers one observes the lowest viscosities in the case of the swollen gels, because in this state the long chain molecules make use of their internal flexibility and according to Eyring and Meyer "move in segments." This movement has been called by Kuhn the micro-Brownian movement; its time of relaxation is between 10^{-3} and 10^{-6} seconds, depending upon the flexibility of the chains and upon the forces between them. It is responsible for the irreversible flow in such systems during extension.

If one removes the solvent of a swollen gel and produces orientation and crystallization by additional mechanical procedures, such as stretching, pressing or rolling, one passes to systems in which much larger unities—bundles of chains or micellae—are the elements which move if the sample flows. Hence the times of relaxation increase up to 10^1 to 10^5 seconds and the samples behave as we know from dry wool, cotton, rayon or hemp fibers. The larger the crystallized areas are, the larger become the particles, which have to move, and the higher gets the viscosity of the sample.

If the time of relaxation finally exceeds 10^5 or 10^6 seconds, the material is completely elastic and we have returned to the considerations of the first paragraph.

In connection with this short description of the pure elastic behavior and the pure flow, we shall now start to discuss the super position of both properties.

THE MECHANICAL BEHAVIOR OF HIGHPOLYMERS

If one takes a sample of a highpolymer such as a wool or cotton fiber, a film of rubber or cellulose acetate, or a cylinder of polystyrene or shellac, and subjects it to a stress or shear, subsequent measurement of the mechanical deformation as a function of time shows in general the following behavior:

a) There is a sudden elongation, El_0 , which is almost completely reversible, if the stress is released after a sufficiently short time (fig. 1, distance AB).

b) Immediately after this sudden jump in length, the sample starts to flow according to a certain initial internal viscosity (slope of the flow curve at the point B in fig. 1). This flow is comparatively rapid in the beginning (point B) and slows down during the experiment; the material undergoes a certain reinforcement while it flows.

c) At the point C this reinforcement is complete and from now on the sample flows with a constant (final) internal viscosity as represented by the final slope of the flow curve between C and D . This flow goes on until the stress is released or if this is not done until the sample breaks.

In order to describe this behavior quantitatively it is necessary to combine elasticity with flow. This can be done in different ways starting with simple cases

and passing to always more and more complicated ones. Maxwell (1867, 1868) has first treated the case of one elastic and one plastic mechanism switched *in series*. In this case one needs one modulus of elasticity and one modulus of flow (viscosity) to describe the behavior of the material. One gets a sudden elongation and a linear flow, but no reinforcement.

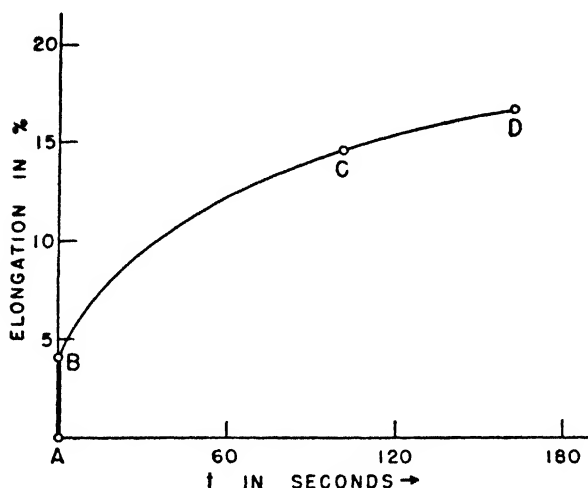


Figure 1. Elongation of a Highpolymer as function of time.

forcement. This means that this type of combining elasticity with flow leads to an interpretation of the parts *AB* and *CD* of the flow curve in Figure 1, but does not explain the piece *BC*. On the other hand, if one switches one elastic and one plastic element *parallel* to each other one gets, according to Maxwell, the part *BC*, which describes the reinforcement. This reinforcement is due to the fact that the plastic elements in the sample gradually release and load up the elastic elements with more and more tension so that the flow slows down more and more.

It seems therefore that one needs two elastic and two plastic elements in order to explain the behavior of highpolymers from the point of view of their mechanical properties. Recently B rgers, H uwink, Jenkel, Holzm ller, Roetger, and Bennewitz have followed this line of investigation and J. Press (unpublished) has undertaken a systematic study of the behavior of a series of highpolymers, such as wool, cotton, viscose, cellulose acetate, etc.

The combination of two moduli of elasticity and two times of relaxation (two viscosities) leads to an equation of the general type

$$El = El_0 + At + B(1 - e^{-t/\lambda})$$

In this expression *El* denotes the total elongation of the sample which is measured as a function of time; *El*₀ is the sudden, elastic elongation at the beginning; *A* characterizes the final flow, while $B(1 - e^{-t/\lambda})$ describes the part *BC* in Figure 1. Obviously *El*₀ and *A* are connected with the elastic and plastic elements in series, while the bracket expres-

sion takes care of the two elements, which are switched parallel. The four constants *El*₀, *A*, *B* and λ allow the calculation of the four quantities which one needs to describe the behavior of the system. Press has found that in some cases an equation of the above type actually describes the experimental curves with fair approximation. This means that in such cases the reinforcement is due to the gradual unloading of the viscous elements and to the transfer of the stress to the elastic mechanism.

However in other cases it seems that there was more reinforcement actually observed than the above mechanism would predict and that the pure exponential bracket expression in (2) would account for. It seems that in such cases additional crystallization takes place and that the relative amount of the two mechanisms *E*₁, λ_1 and *E*₂, λ_2 does not remain constant during the extension, but changes in favor of the mechanism with the higher time of relaxation.

If one has once determined the quantities *E*₁, λ_1 , *E*₂ and λ_2 , one may go back to the four cases of paragraph 2 in order to correlate them with a particular molecular mechanism. In the case of rubber, for example, Kuhn has shown, that there is one mechanism with an elastic modulus around 10^{10} and a time of relaxation around 10^{-3} seconds, while the other has a modulus of 10^6 dynes per sq. cm. and a time of relaxation of 10^5 seconds. In other cases like wool, cellulose acetate, polystyrene etc., the conditions seem to indicate that the two mechanisms are not independent from each other, but are connected by a first order phase transition. However it would be perhaps premature to enter into more detailed discussion until the present experimental material is more completely evaluated along the lines indicated in this brief comprehensive article.

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DISCUSSION

MULLER: Do Astbury's ideas on the stretching of wool fit into this picture?

MARK: Yes, they do. But Astbury describes mainly the elastic part of the curve. He explains this elastic part from the point of view that the chain is folded in the contracted state and extended in the elongated state. He thinks that in the case of proteins there are not only entropy differences but also energy differences. In wool and protein, therefore, the folding would be governed by forces rather than by statistics. On the other hand, the folding of rubber is due to the tendency to go to a more random state.

HOECKER: The whole process seems not to be completely reversible. Is it true then that in the cycle there is a small increase of the entropy?

MARK: This would be true if no heat were produced. But during stretching of rubber, 3.99 calories per gram are evolved. This is the heat of crystallization.

HOECKER: Is there any implication that there is a relation between irreversibility of the process and ultimate failure of the material? In my experiments on plastics, it is apparent that the material fails after a number of experiments.

MARK: It may be possible to describe such a relation as follows: In the beginning we have a certain amount of the material in the amorphous and a certain amount in the crystallized state. If we stretch it, we get a little more crystalline material and the chains in the amorphous areas are slightly more stretched than normal. A certain amount of crystallization has taken place and the amorphous parts are stretched. This corresponds to a two-fold entropy loss: some chains have lost all their entropy, others have been partially straightened. If one repeats the experiment one gets more additional crystallization until finally the sample breaks.

HUSKINS: Is there any possible suggestion of a relation between molecular structure and gross structure? If there is, wool gives a better chromosome analogy than rubber in that it crimps, etc.

MARK: One point is the average length of the chains; it seems that strength is only developed if the chains exceed 60 to 90 links. If the material is degraded below this, it gets very brittle. Protein

chains are probably much longer than this and therefore develop considerable strength. Another point is that cross-linking or branching of the chains influences hardness, impact strength and elasticity. If the chains are tightly cross-linked one gets materials of the type of hard rubber.

HUSKINS: Branching is another factor. Is there a more direct relation between molecular and gross structure for example in wool of different crimp, or are we talking of incommensurables?

MARK: Not very much is known about the origin of crimp in wool, but crimping of cellulose fibers can be obtained by appropriate adjustment of temperature, spinning bath, spinning speed and other conditions. This crimp, however, is not as permanent as the one in wool.

FANKUCHEN: How can we talk about crimping as related to molecular structure when we do not know the molecular structure? Astbury's old theory of the crimping of wool can now be abandoned since he has published in a recent number of *NATURE* a new theory for the structure of the folded protein chain.

MARK: Wool is a very complicated copolymer and it would certainly be difficult to correlate crimp directly with the molecular structure, even if we knew it.

MULLER: Without reference to the Astbury mechanism, do you not still have good evidence that it is regularly folded?

FANKUCHEN: We must consider it folded in some way. Some regularity is indicated by the X-ray pattern.

MULLER: It must be regular.

FANKUCHEN: Yes, since you get a picture. It is like the kinetic theory of gases—you can look at it statistically and explain a lot but you cannot account for every molecule.

MULLER: Is the folding in rubber regular or irregular?

MARK: There is good evidence that it is practically irregular. Therefore one feels entitled to apply the laws of probability. As soon as one suspects that there is no complete irregularity, as perhaps in proteins, polyesters or polyamides, it is necessary to take care of these irregularities.

WRINCH: In listening to the brilliant paper by Dr. Mark, I have been struck by the many ways in which the data which his researches have yielded are relevant to the problem we have had under discussion in this Symposium. We have been particularly concerned with studying all the possible ways in which information regarding the structure of chromosomes can be elicited by means of techniques belonging to any field of experimental or theoretical science. Now Dr. Mark has shown, to take one example, how elongation/time curves for synthetic or natural polymers can be interpreted so as to yield information regarding the spatial arrangement of the atoms. Here then is a situation in which macroscopic data can be used directly to ob-

tain insight into atomic architecture. I would emphasize particularly the interesting difference which obtains, from this point of view, between polymers in which the interchain attractions are necessarily of the van der Waals type and polymers in which (as in the case of nylon) there is, in addition to such attractions, the stereochemically more significant attractions between the polar groupings. Since there is, as Dr. Mark has shown, a distinctive difference in the dependence of such elongation/time curves on temperature, there would seem to be an opportunity in the future for building the bridge on these lines between the atomic architecture of chromosomes which we wish to understand and the microscopic behavior of chromosomes which present techniques allow us to study.

In this Symposium we have, of course, been very much concerned with forces between different molecular units in general. In particular we have been much concerned with ways in which the specificity (which must for genetic reasons reside in the chromosome structure) can be detected directly by experimental means. Now a whole family of nylons of the composition $\text{—NH—CO—(CH}_2\text{)}_n\text{—NH—CO—}$, in which n is varied, would perhaps be capable of providing a very simple model for atomic specificity. Also, the various synthetic rubbers, where there are a variety of hydrocarbon units, might be used as a model for specificities in which only van der Waals attractions play a part. May I therefore ask Dr. Mark whether there would be any simple way in which mixtures of different nylons or mixtures of different synthetic rubbers could be studied, as a first attempt at correlating atomic specificities with easily measured macroscopic parameters? If there were, we might have some indications as to methods by which the more delicate specificities of our biological materials could be brought under direct observation in future studies.

MARK: The best possibility may be a thermoelastic investigation. There is a distinct difference between compounds such as polyesters or polyamides and rubber; in the latter, one has a chain with weak, continuous lateral forces, in the former one has chains with distinct, strong sticking points. If such a material, having such sticking points, is extended to about one or two percent, it shows reversible elasticity with a modulus of about 10^7 dynes per sq. cm. but with a negative temperature coefficient. On the other hand, the elastic modulus of rubber increases with temperature. This agrees

with the idea of E. Mack, that in the unstretched state the chains are folded together, with the sticking points in particularly favorable positions. To extend such a material, one must first lift the chains out of the potential holes of the van der Waals forces and put them in a position to show kinetic elasticity. Such material has first a negative and later a positive temperature coefficient of the modulus of elasticity.

WRINCH: There is a chance that such techniques would be applicable to biologically active materials provided the temperature range were not too great. Would you be able to detect a difference in nylons?

MARK: There are no published measurements of this type accessible in the case of nylon, but one has some kind of general knowledge of how copolymerization influences elasticity. If one wants to change the elastic properties of a given substance by copolymerization, one usually has to go to 15-20 percent weight percent of the second component in order to obtain a measurable effect.

HOECKER: There seem to be two moduli of elasticity and two of flow. Does one modulus represent one phase and the other modulus represent another phase of the system?

MARK: It would be a satisfactory situation if the two moduli of elasticity and of flow would correspond to the two phases which one finds by X-ray measurements, but there is no proof for this coincidence yet available.

HOECKER: As the curve proceeds, one goes into the other and finally we have only one. Dr. Wrinch wants to write an equation of the curve with moduli so you can assign components to each. Has this been done?

MARK: Yes, it has been tried and the results are not too discouraging.

COLE: I would like to ask about relaxation. Why does rubber, if stretched, unstretch again?

MARK: As far as our present knowledge goes the unstretching in the range up to 200 percent is not due to molecular forces. In the unstretched condition, the random molecular movement keeps the chains in a slightly folded condition. If one extends the chains, the arc stretches but still gets impacts from all directions and these irregular thermal impacts tend to curl the chains up again.

COLE: Does this explanation still hold if some of the material is crystalline?

MARK: Yes, it holds for the part of the material which is still in the uncrystallized state.

PROTEOLYTIC ENZYMES AS SPECIFIC AGENTS IN THE FORMATION AND BREAKDOWN OF PROTEINS

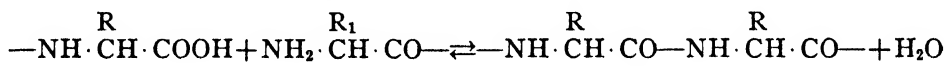
JOSEPH S. FRUTON

It has been suggested that genes are nucleoproteins that can act as enzymes in the formation of duplicates of their own structure and thus are able to perpetuate themselves through successive nuclear divisions (Gulick, 1938). In this property of auto-synthesis, genes resemble two other nucleoproteins—tobacco mosaic virus and bacteriophage—which apparently also act as enzymes in catalyzing the creation of replicas of their own structure from the materials provided by the host organism (Stanley, 1940; Northrop, 1938). Furthermore, there seems to be no doubt that the biological formation of the innumerable other proteins encountered in nature also involves the participation of enzymes. Although the importance of enzyme reactions in protein synthesis has been generally acknowledged, experimental progress in this field has been laboriously slow. The principal difficulty is that the final product in protein synthesis is a large molecule of so complex a structure that it is still impossible to write down the structural formula of a single well-defined protein. However, there are a few facts regarding protein structure of which one can be reasonably certain. It seems firmly established that in the formation of proteins, linkages between amino acids are formed whereby the carboxyl group of one amino acid is joined to an amino group of a second amino

acid to produce the so-called peptide bond. Consequently, in discussing the biological synthesis of proteins we must limit ourselves, for the present, to a consideration of the agents that are capable of synthesizing peptide linkages and leave the consideration of the formation of other types of linkages in proteins for a day when they are more perfectly understood.

However, even when the study is limited to the synthesis of peptide linkages there still exists the difficulty that in the synthesis of proteins there must occur the enzymatic catalysis of numerous simultaneous and consecutive chemical reactions. It will be a formidable task to unravel the complex dynamics of protein synthesis if we do not use simple models that permit us to study the synthesis of single peptide linkages under isolated experimental conditions. The purpose of this paper is to consider some of the evidence on the mechanism and specificity of the synthesis of peptide linkages in proteins, that has been obtained through the study of the synthesis of peptide linkages in simple peptides and peptide derivatives (Bergmann and Fruton, 1941).

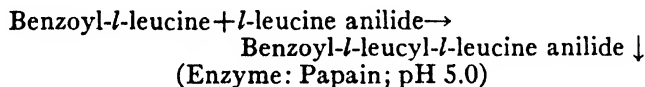
The only enzymes that are known to synthesize peptide linkages belong to the group designated proteolytic enzymes. These enzymes catalyze the reaction:



acid to produce the so-called peptide bond. The high molecular weight of even the smaller proteins requires that a large number of such peptide linkages be present in a protein molecule. On analysis of an individual protein as many as twenty different amino acids may be isolated, and it must be concluded that each of these structural units is distributed in the peptide chain to give a complex linear pattern. Dif-

ferent proteins contain different proportions of various amino acids and the chemical, physical, and biological properties of each protein are determined by the nature and position of the amino acids in the peptide chain. There is evidence from physico-chemical data that the polypeptide chains undergo folding in a rather specific manner to yield "globular" protein molecules (Neurath, 1940). However, the nature of the linkages that are involved in producing the specific shape of the protein molecule is not known, although this question has been the sub-

ject of extensive and stimulating speculation. Consequently, in discussing the biological synthesis of proteins we must limit ourselves, for the present, to a consideration of the agents that are capable of synthesizing peptide linkages and leave the consideration of the formation of other types of linkages in proteins for a day when they are more perfectly understood.



However, the nature of the linkages that are involved in producing the specific shape of the protein molecule is not known, although this question has been the sub-

It must be emphasized that this is an equilibrium reaction and that the function of the enzyme is to accelerate the attainment of the equilibrium concentrations no matter from what side of the equation one starts. An example of the enzymatic synthesis of a peptide linkage is the reaction (Bergmann and Fraenkel-Conrat, 1938):

However, the nature of the linkages that are involved in producing the specific shape of the protein molecule is not known, although this question has been the sub-

It is possible to reverse the hydrolytic reaction and perform a synthesis if the effective concentration of the peptide is continuously kept at a value much below this equilibrium concentration of the peptide. In the model synthesis presented above, the concentration of the synthetic product was kept below the equilibrium concentration through insolubility. The effective concentration of a synthetic product may be kept below the equilibrium concentration in other ways. For example, acetyl phenylalanylglycine and the dipeptide glycylleucine are combined enzymatically to form an acetyl tetrapeptide, acetyl phenylalanylglycylglycylleucine. This acetyl tetrapeptide is hydrolyzed at the terminal peptide linkage and leucine is split off. In other words, as fast as some of the synthetic product is formed, it is subjected to another enzymatic action that removes it from the reaction (Behrens and Bergmann, 1939).

In living systems, in addition to factors of insolubility or removal by a subsequent proteolytic process, the intervention of other driving forces may favor peptide synthesis. One mechanism may be

mechanical removal of the synthetic product by the circulatory system; another may be the stabilization of the synthetic product by combination with some non-protein material such as nucleic acid. Furthermore, more complex interrelationships with other enzyme systems such as those concerned with oxidation-reduction might, under proper metabolic conditions, yield the necessary driving force for peptide synthesis.

As was mentioned before, the process of protein synthesis apparently involves the creation of a linear pattern in which each of the various amino acids is located at certain positions in the peptide chain. Since a given cell under normal conditions synthesizes similar protein molecules continuously and reproducibly, there must exist a mechanism for the selective introduction of amino acids into the protein. Thus the enzymes that catalyze the formation of peptide linkages must have such a specificity that at each step of the synthesis the required amino acid will be introduced into the peptide chain. It is not sufficient, therefore, to show that the proteolytic

TABLE 1. CLASSIFICATION OF PROTEOLYTIC ENZYMES

Sub-group	Examples of enzymes	Typical substrates	Requisite groups in peptide chain
A. Peptidases (Exopeptidases*)			
Aminopeptidases	Intestinal aminopeptidase	<i>l</i> -Leucine amide	$\begin{array}{c} \text{R} \\ \\ \text{NH}_2 \cdot \text{CH} \cdot \text{CO}-\text{NH} \cdots \\ \updownarrow \\ \text{R} \\ \\ \text{NH}_2 \cdot \text{CH} \cdot \text{COOH} + \text{NH}_2 \cdots \end{array}$
Carboxypeptidases	Pancreatic carboxypeptidase	Carbobenzoxycarbonyl <i>l</i> -phenylalanine	$\begin{array}{c} \text{R} \\ \\ \cdots \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{COOH} \\ \updownarrow \\ \cdots \text{COOH} + \text{NH}_2 \cdot \text{CH} \cdot \text{COOH} \end{array}$
B. Proteinases (Endopeptidases*)			
Carbonyl proteinases	a. Trypsin	Benzoyl- <i>l</i> -arginine amide	$\begin{array}{c} \text{R} \\ \\ \cdots \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{CO}-\text{NH} \cdots \\ \updownarrow \\ \text{R} \end{array}$
	b. Pepsin	Carbobenzoxycarbonyl- <i>l</i> -glutamyl <i>l</i> -tyrosine	$\begin{array}{c} \text{R} \\ \\ \cdots \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{COOH} + \text{NH}_2 \cdots \end{array}$
Imidoproteinases	Chymotrypsin	Carbobenzoxycarbonyl- <i>l</i> -tyrosyl glycineamide	$\begin{array}{c} \text{R} \\ \\ \cdots \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{CO}-\text{NH} \cdots \\ \updownarrow \\ \text{R} \\ \\ \cdots \text{COOH} + \text{NH}_2 \cdot \text{CH} \cdot \text{CO}-\text{NH} \cdots \end{array}$

* Bergmann and Fruton, 1939.

enzymes can catalyze the synthesis or hydrolysis of peptide linkages, but it is also necessary to determine whether they exhibit the requisite highly developed specificity in performing these reactions.

The specificity of a proteolytic enzyme is characterized by the relative rates at which the peptide bonds are hydrolyzed or synthesized in a number of substrates. In order to determine the specificity one examines the effect of changes in the chemical structure of a typical substrate and in that way one can decide whether the group that has been replaced is essential or non-essential for the enzymatic action. In the specificity studies to be discussed below, the information was largely obtained in experiments on the enzymatic *hydrolysis*, rather than the enzymatic *synthesis*, of peptide bonds. The assumption that the action of an enzyme is to catalyze the attainment of an equilibrium requires that the specificity of hydrolysis be the same as that of synthesis. In every case where this assumption has been tested, it has been found to be correct.

One of the striking characteristics of protein metabolism is the division of proteolytic labor among a large number of peptide-splitting enzymes. These proteolytic enzymes may be distinguished from one another through differences in their specificity, the pH at which they act optimally, the influence of added substances, etc. The classification of these enzymes on the basis of their specificity (Fruton, Irving and Bergmann, in press) enables one to fit most of the known proteolytic enzymes into one of two broad groups (table 1).

In the first group are the enzymes that are restricted in their action to peptide bonds at the end of a peptide chain and are thus usually designated peptidases. One sub-group of these enzymes selectively attacks the chain at the peptide linkage adjacent to the amino end of the chain while another sub-group attacks the chain at the peptide linkage adjacent to the carboxyl end of the chain. Apparently the enzymes require the presence of a free amino or free carboxyl group adjacent to the peptide bond. For this reason these two sub-groups are called aminopeptidases and carboxypeptidases respectively. The amino- and carboxypeptidases cannot split peptide linkages that are more centrally located in the peptide chain. Since proteins contain very few terminal peptide bonds, these enzymes cannot split proteins.

The second broad group of peptide-splitting enzymes is capable of splitting linkages that are not adjacent to a terminal group—however, the sensitive peptide linkage must be adjacent to another peptide bond. Since these enzymes are capable of hydrolyzing and synthesizing central peptide bonds, they can hydrolyze and synthesize peptide bonds in proteins. The members of this group are usually designated proteinases. Here again we have two sub-groups, depending on whether the sensitive peptide linkage is on the left side or the right side (as written in this paper) of the additional essential peptide

bond. Examples of the first sub-group are pepsin and trypsin, while chymotrypsin is a representative of the second sub-group.

In this classification we have considered only specificity differences based on the chemical structure of the peptide chain itself. We know, however, that within each group and sub-group further differences in specificity are exhibited. For example, both trypsin and pepsin are in the same sub-group of the proteinases, although it has been known that trypsin will attack peptide linkages in some proteins (proteins, histones) that pepsin will not split. This difference in specificity shows that although these two enzymes apparently require the same structural characteristics of the peptide chain, other structural factors are of paramount importance in determining whether a given peptide bond will be split by either of these enzymes. Specificity studies on trypsin and pepsin, as well as other proteinases, by means of simple specific peptides have shown that the nature of the side chains that jut out from the chain play an important role. These side chains vary with each amino acid and exert a profound influence on the properties of the peptide linkages in their vicinity. For example, trypsin hydrolyzes the simple substrate benzoyl-*L*-arginineamide (Bergmann, Fruton and Pollok, 1939). The side group that juts out is the long aliphatic chain bearing a guanidine group on the end. If this side chain is replaced by the basic side chain of lysine, the resulting benzoyl-*L*-lysineamide is still hydrolyzed, but more slowly than the arginine compound (Hofmann and Bergmann, 1939). However, substitution of the side groups of amino acids other than arginine or lysine completely abolishes the hydrolysis by trypsin.

Similarly, in the case of pepsin, the presence of an aromatic amino acid such as tyrosine or phenylalanine was found to be essential for the enzymatic action (Fruton and Bergmann, 1939). The best simple substrate was of the type carbobenzoxy-*L*-glutamyl-*L*-tyrosine. Of the two amino acids present in this substrate, only the aromatic amino acid is indispensable; the glutamic acid may be replaced by other amino acids such as glycine or tyrosine. However, the resulting substrates are split much more slowly than when glutamic acid is present. Apparently pepsin action is favored by the presence of acidic groups in the substrate; hence the combination of glutamic acid and tyrosine results in the best substrates for this enzyme.

In this connection it is of interest to mention that pepsin itself is a rather acidic protein, having an isoelectric point at a pH less than pH 1 (Herriott, Desreux and Northrop, 1940). On the other hand trypsin, which selectively hydrolyzes peptide linkages in basic substrates, itself is a basic protein with an isoelectric point at about pH 7 to 8 (Northrop, 1939). One may speculate as to whether there is a direct relation between the structure of the enzyme, as expressed in the nature of the amino acid side groups that jut out from its peptide chain and the

nature of the amino acid side groups in the substrate. Does a proteolytic enzyme hydrolyze or synthesize a peptide linkage only when it finds an arrangement of amino acids in the substrate that mirrors the amino acid pattern of the enzyme? The answer to this question would be of interest for the problem of the autosynthesis of nucleoproteins such as tobacco mosaic virus or genes, as well as the general problem of protein synthesis. Unfortunately, thus far there is no sound basis for such speculations, but it may be hoped that future experiments will throw some light on the matter.

Enough has been said about the specificity of pepsin and trypsin to indicate that they show a precise and rather selective mechanism for the hydrolysis of peptide linkages involving particular amino acid residues. The studies on other proteolytic enzymes, notably chymotrypsin (Bergmann and Fruton, 1937) and carboxypeptidase (Hofmann and Bergmann, 1940), show that this dependence of specificity on the nature and sequence of the amino acid

residues is a general characteristic of proteolytic enzymes.

The enzymes that have provided the clearest information on specificity have been those found in the gastrointestinal tract, namely pepsin, trypsin, chymotrypsin, and pancreatic carboxypeptidase. This is due to the fact that these proteolytic enzymes have been obtained in crystalline form by Northrop, Kunitz, and Anson (Northrop, 1939). In the course of protein metabolism these proteolytic enzymes are probably primarily concerned with the degradation of the ingested foreign proteins into amino acids and small peptides. These split-products are transported by the blood to the tissues where they are utilized for the synthesis of the characteristic proteins. It is therefore important to know something about the specificity of the intracellular proteolytic enzymes found in animal tissues.

The fact that extracts of animal tissues have powerful proteolytic enzymes called "cathepsins" has been known for a long time. However, the task of

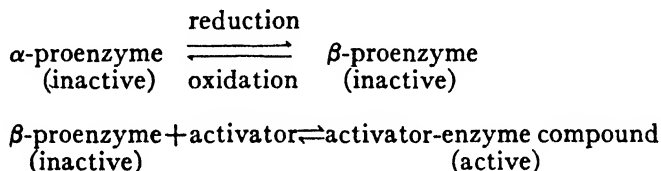
TABLE 2. PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

Cathepsin	Specific substrates	Activation behavior
I	$\begin{array}{c} \text{COOH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{COOH} \\ \\ \text{C}_6\text{H}_5\text{OH} \end{array}$ Carbobenzoxyl-L-glutamyl-L-tyrosine	Requires no added activator.
II	$\begin{array}{c} \text{NH}_2 \\ \\ \text{C}=\text{NH} \\ \\ \text{NH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{C}_6\text{H}_5 \cdot \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{CO}-\text{NH}_2 \end{array}$ Benzoyl-L-arginineamide	Requires activator—sulfhydryl compounds (e.g., cysteine, H ₂ S). Ascorbic acid does not activate.
III	$\begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \diagdown \quad / \\ \text{CH} \\ \\ \text{CH}_2 \\ \\ \text{NH}_2 \cdot \text{CH} \cdot \text{CO}-\text{NH}_2 \end{array}$ L-Leucineamide	Requires activator—sulfhydryl compounds or ascorbic acid.
IV	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2 \\ \\ \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CO}-\text{NH} \cdot \text{CH}_2 \cdot \text{CO}-\text{NH} \cdot \text{CH} \cdot \text{COOH} \end{array}$ Carbobenzoxylglycyl-L-phenylalanine	Requires activator—sulfhydryl compounds. Ascorbic acid does not activate.

purifying the intracellular enzymes has proved rather difficult, so that we do not as yet have any of them in a pure form. Despite this, the sharp specificity of the proteolytic enzymes enables us to select simple substrates that will be hydrolyzed by only one enzyme present in a mixture of enzymes. With the use of such substrates and also by means of well-known techniques such as dialysis, precipitation by salt, partial inactivation by heat or acid, it has been possible to identify in aqueous extracts of several tissues at least four different proteolytic enzymes (Fruton, Irving and Bergmann, 1941, and in press). These are listed in Table 2. It will be noted that Cathepsins I and II are proteinases and consequently are able to act at peptide linkages in the interior of peptide chains. Cathepsin III is an aminopeptidase and Cathepsin IV is a carboxypeptidase. Furthermore, these enzymes show the sharp selectivity based on the nature of the amino acid residues in the peptide chain, such as was discussed above for trypsin and pepsin.

It is likely that additional proteolytic enzymes will be identified in tissue extracts in the course of future work, but it is already clear that animal tissues are equipped with a variety of enzymes that act at peptide linkages with high specificity. It is not yet known whether these enzymes are all present in each cell of a tissue or whether the mixture is produced on destroying the cells and extracting the enzymes. This question may perhaps be answered through the application of micro methods such as those developed by Linderstrøm-Lang and Holter for the study of intracellular enzymes.

It will be noted in Table 2 that certain of the intracellular proteolytic enzymes require an additional substance, such as cysteine or glutathione, to exhibit full enzymatic activity. The mechanism of this activation is still under investigation; at the present time the experimental facts are best explained by the following picture of the activation process. An inactive proenzyme is converted by traces of reducing substances into a second form that is also proteolytically inactive. However, the second form differs from the first in that it can combine with substances such as HCN, H₂S, cysteine, glutathione, etc., to become an active proteolytic enzyme (Irving, Fruton and Bergmann, 1941). This mechanism may be represented schematically as follows:



The formation of the active enzyme requires relatively large concentrations of the activator. Not all compounds that are capable of forming addition compounds with the β -proenzyme can also convert the α -form to the β -form. An example is HCN which, even in high concentrations, cannot reduce the

α -form to the β -form. If a trace of cysteine is added, however, the resulting β -proenzyme can combine with HCN to form an active enzyme. Of particular importance in this scheme is the fact that the enzyme-activator compounds that are formed are reversibly dissociable. If a volatile activator such as HCN or H₂S is employed, it is possible to inactivate the enzyme by simply removing the activator under reduced pressure.

The fact that the activation process involves the formation of reversibly dissociable activator-enzyme compounds raises the question as to whether the specificity of an activated enzyme is influenced by the nature of the activator that participates in the activation. It may be regarded as probable that the specificity of an activated enzyme is modified by the nature of the activator. Experiments now in progress may soon give a definite answer.

Before leaving the question of the activation of the intracellular enzymes it might be mentioned that, as was to be expected, the activated enzymes perform synthetic reactions at the same pH and under the same condition of activation as they perform hydrolytic reactions.

To summarize, while there is no direct experimental proof for the statement that the proteolytic enzymes are responsible for the biological synthesis of proteins, it must be admitted that at the present time these enzymes are the only agents that are known to catalyze the synthesis of peptide linkages. Furthermore, the proteolytic enzymes, because of their sharp specificity, are able to accelerate selectively only a few of many possible equilibrium reactions. Clearly the biological synthesis of the specific pattern of amino acids requires the existence of a mechanism whereby the sequence of peptide syntheses is directed into specific channels. In addition, the specificity may be modified slightly by the absence or change in concentration of other normal cell constituents and thus result in a change in the sequence of synthetic reactions. Finally, in order to exhibit synthetic activity in a homogeneous medium the reactions catalyzed by proteolytic enzymes must be coupled with another equilibrium reaction. This coupling provides the necessary driving force for the synthesis but does not affect the specificity of the reaction.

The fact that proteolytic enzymes exhibit all these

properties *in vitro* makes it more likely that they play a central role in the course of *in vivo* synthesis of proteins. There is much difficult work ahead to determine more precisely the function of the proteolytic enzymes as well as of the other enzyme systems in protein synthesis. It may be hoped that the

results presented in this paper will serve as a starting point for fruitful future studies.

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DISCUSSION

ANSON: 1) Why do not exopeptidases split off the amino acids of a protein one by one?

2) Is there any relation between the affinity of a proteolytic enzyme for its substrate and the rate at which the substrate is digested by a given amount of enzyme when all the enzyme is combined with substrate?

3) Is it possible that in the second stage of the activation of a proteolytic enzyme by cysteine or cyanide, the activator does not combine with the enzyme but combines with and removes heavy metals which inactivate the active SH form of the enzyme? As long as one is dealing with complex mixtures of substances containing unknown impurities it is difficult to be sure of the mechanism of activation.

FRUTON: 1) The fact that exopeptidases do not split proteins indicates that there are few, if any, terminal amino acids in well-defined proteins.

2) There is a relation between the affinity of the enzyme for its substrate and ease of digestion. The Michaelis equation expresses the fact that the rate of hydrolysis is a function of the concentration of the enzyme-substrate compound.

ANSON: I meant to ask whether you found any correlation between the affinity constant calculated

from the Michaelis equation and the rate of hydrolysis of the substrate-enzyme compound.

FRUTON: Determinations of the Michaelis constants have not been carried out for the hydrolysis of our synthetic substrate.

3) I believe that it is unlikely that the reversible inactivation is due to the liberation of a metal inhibitor. It is a possibility that can be tested experimentally.

FURCHGOTT: Different maximum reaction rates of a single enzyme with excesses of different activators would be evidence against the proposed action of heavy metals in the activation process.

FRUTON: Recent data show that the value of the velocity constants for the hydrolysis of benzoyl arginine amide by activated pepsin depends on the nature of the activator employed.

WRINCH: I have been very much interested in the report of Dr. Fruton of the very elegant and successful studies on the specificities of many enzymes directly relevant to the synthesis and breakdown of polypeptide systems. May I ask whether there is any evidence of foreign ions playing any part in the interaction of the acidic protein pepsin and its substrates containing acidic groupings? It was my thought that an enzyme-substrate complex of such a kind would be greatly facilitated by the presence of ions, somewhat after the manner of the atomic arrangement in the metallic hydroxides.

FRUTON: There is no evidence, to my knowledge, that the interaction of pepsin and its acidic substrates involves the participation of foreign ions.

WRINCH: I gather from the account given by Dr. Fruton that the enzymes which are capable of splitting the end residue from a polypeptide chain have never been shown to have any effect upon intact proteins.

FRUTON: The enzymes we call exopeptidases have no effect on well defined proteins.

WRINCH: I gather then that Dr. Fruton deduces that the proteins in question, which he assumes consist of single polypeptide chains, do not possess open ends, in other words that they are not the open chains postulated by Emil Fischer and others but are rather closed chains or rings, such as were postulated in the cyclol theory. If this is his deduction, I think implications of some importance follow. In the procedure of titration of proteins, it was, I believe, uniformly found that there was little or no room for postulating any α -amino or α -carboxyl groups. From this fact comes the assumption of a single polypeptide chain rather than many in one protein molecule. But if we are now faced with replacing the open chain by a closed chain or ring, no necessity for a single such (closed) chain remains. We can as easily postulate several closed polypeptide chains, i.e., rings in a single molecule. I would venture to draw attention to the elegant way in which the postulate that a single protein molecule may be made up of a number of interlinked polypeptide rings (which is the essence of the fabric theory of

protein structure) fits in with the interesting results of Dr. Fruton according to which different enzymes are required for the condensation of different species of amino acids. On this picture, it would be the task of each of the enzymes involved to synthesize its contribution to the fabric of the new protein, consisting of special varieties of amino (or imino) acids, perhaps in the form of a hexapeptide or other small ring (possibly in cyclized form), the protein itself being the result of tautomeric association of such fragments together.

GREENSTEIN: May I also emphasize electrochemical specificity? At the pH range where pepsin is active, all NH_2 groups are ionized. With trypsin all the carboxyls are ionized.

The fact that pepsin will split tyrosine from carbobenzoxy glutanyl tyrosine permits the idea of free polypeptide chains. What is the evidence of Calvery on the isolation of amino acids after the action of pepsin?

FRUTON: Northrop observed that pepsin preparations, when allowed to stand, deposited tyrosine crystals formed through the action of active pepsin on denatured pepsin. Calvery and Damosaran have

also shown that pepsin liberates free amino acids from proteins. From the data with synthetic substrates it is clear that pepsin can hydrolyze peptide linkages in the interior of peptide chains and also, under certain conditions, hydrolyze off terminal tyrosine residues.

ANSON: One could find out whether a native protein has any free terminal amino acids by coupling all the free amino and carboxyl groups of the native protein with suitable groups, and then hydrolyzing the protein and seeing whether any of the monocarboxylic or monoamino acids in the hydrolysate had combined with the coupling reagents. Has this ever been done?

FRUTON: I do not know of any successful attempts to show this. If any terminal amino acids are present at all, the amount of acylated amino acids would be so small that their isolation would be very difficult. Gurin and Clarke used this method for the free amino acids in gelatin. They reported that at least half of the amino groups could be attributed to the η -amino group of lysine and not more than 0.5 percent could be attributed to α -amino groups.

THE NATIVE PROTEIN THEORY OF THE STRUCTURE OF CYTOPLASM

DOROTHY WRINCH

I have selected cytoplasm as the subject of my contribution to this symposium for various reasons. (1) The cytoplasm has aptly been called the *substratum of life* (Frey-Wyssling, 1940) since it is the medium in which many of the processes of living matter take place. Its study is therefore of the first importance for physiology and biology in general, and for cytogenetics in particular, and as such merits a place in our deliberations. (2) There now seems to be a consensus of opinion that the essential characteristic of cytoplasm is its structure. Thus it is reasonable to hope that the policy of applying to biological materials the newer knowledge of structure chemistry against a background of classical and modern geometrical concepts (Wrinch, 1934, 1936) may yield results in this new field. (3) The cytoplasm contains, in addition to a very large amount of water and certain other substances, such as carbohydrates, fats and salts, some native protein. A fairly complete picture of the nature and characteristics of native proteins has been obtained in recent years from studies in physical chemistry, crystallography and enzyme and immunochemistry. It would, therefore, seem of interest, and even of urgent importance, to consider the problems of cytoplasmic structures in the light of this newer knowledge. This seems necessary especially in view of the fact that recent discussions have uniformly adopted as axiomatic the older picture of protein structure, antedating Svedberg's work and based upon nothing more relevant than certain ideas derived from studies of silk, wool, feather quill, nail, scales, spines, horn, tendons and cartilage, proteins which appear to play little part in the essential life processes. In the cytoplasm, if anywhere, the proteins must be presumed to be in their native state.

THE PRESENT DAY PICTURE OF THE NATIVE PROTEIN

The present day picture of the native protein is the resultant of ideas and facts stemming from many different fields of experimental work and from the application of geometrical concepts. A vivid description of the native protein as it now appears has been given by Svedberg (to whose monumental labors with scores of native proteins this newer point of view is in part due) on more than one occasion. "Investigation along different lines" says Svedberg in summing up his work on proteins at the Royal Society discussion on the protein molecule in 1938, "has given the result that the proteins are built up of particles possessing the hallmark of individuality and therefore are in reality giant

molecules. We have reason to believe that the particles in protein solutions and protein crystals are built up according to a plan which makes every atom indispensable for the completion of the structure. . . . The vague term 'colloid' for a protein has been replaced by precise information concerning the mass and electrochemical behavior and in certain cases even of the shape and structure of the protein particles in solutions and crystals. These entities appear so well defined that we really feel justified in denoting them as protein molecules" (Svedberg, 1939).

Protein crystallography yields the same picture, of globular bodies carrying R-groups arranged in definite spatial patterns. It is noteworthy that even the driest and most stable of protein crystals, namely insulin crystals, contain water associated with the protein units, so that it appears that in structure systems, of this kind at least, proteins are characteristically accompanied by water. The remarkable fact that native proteins form crystals of high symmetry is well known. The deep significance of the fact that two of the very few so far examined belong to the space group having the highest symmetry, namely the cubic class, whereas among other organic and inorganic crystals, a very much smaller proportion have so high a symmetry, perhaps deserves emphasis. The only reasonable deduction from this fact, in view of the high molecular weights of the proteins, must surely be that protein crystals consist of units which have a very highly organized and specific structure, that (as Svedberg phrases it) native protein units are in reality giant molecules.

For a statement of the situation as the crystallographer sees it, we cannot do better than quote Bernal (1939). The X-ray examination of a number of crystals of native protein has, says Bernal, "revealed important new facts. In the first place, the pictures yielded by protein crystals were of exceptional perfection. They showed large unit cells with a great wealth of reflections and these reflections were found even at comparatively high angles corresponding to such low spacings as 2 Å. This indicated that not only were the molecules of the proteins substantially identical in shape and size, but that they had identical and regular internal structures reaching right down to atomic dimensions." It is specially interesting that even the bushy stunt virus protein—perhaps the nearest thing to a gene protein—with a molecular weight of many millions, appears to have a perfectly definite structure (Bernal, Fankuchen and Riley, 1938).

Other fields of work yield the same picture. Thus

it can hardly be doubted that the protein enzymes, such as pepsin, urease, trypsin, chymotrypsin, have very definite and specific structures, due, we must presume, to a highly organized atomic pattern. Direct evidence that enzymes act by means of rigidly interrelated groups of atoms, in other words that they act in virtue of a specific surface pattern, is offered by the important work of Bergmann and his collaborators (1935) on dipeptidase.

From a quite different field, the field of immunochemistry, we get confirmation of this picture. Thus Marrack, whose great contribution to this subject includes the application of the ideas of structure chemistry to problems of the architecture of the immune bodies, writes of the serum proteins as probably having some areas on their surface which are specially suitable for binding particular molecules (a suggestion which would account for the adsorption of dyes by normal serum proteins). "It is conceivable," he writes (1939), in discussing the relation between normal and modified serum globulins, "that slight alterations in the structure of globulin molecules might give rise to areas with a specific affinity for certain determinant groups. One can compare the effect of the simple change from diamond to graphite packing of carbon atoms on the adsorption of methylene blue and succinic acid." Clearly Marrack feels that the facts of immunochemistry require us to think of the native proteins concerned as having a definite atomic pattern on their surfaces which entails a perfectly specific spatial arrangement of the C_α atoms at which the R-groups are attached to the skeleton of the molecule. He even suggests that it is simply the existence of localized recurring groups of certain R-groups which form the determinant groups of natural proteins, and he talks frequently of "patches" on the surfaces of antibodies, which have affinities to the antigens.

In this communication, therefore, I propose to assume that the native proteins are compact structures having a highly developed individuality and specificity. This compact globular structure must necessarily carry on its surface some characteristic atomic pattern. Physico-chemical studies show that many and perhaps all the R-groups emerge from the surface. The definite architecture, which it is necessary to assume the molecule possesses, then implies that these R-groups are rooted in C_α atoms which are arranged in a definite spatial pattern, leading to a certain rigidity in the molecule, so long as the native configuration remains intact. In my opinion we shall not go far wrong if we think of the native protein units as bounded externally by a rigid shell of atoms, in which each atom has its own correct position and picture a number of R-groups emerging from fixed positions on this shell. With this picture, the type or types of atomic patterns constituting the shell may be taken as characteristic of native proteins, while the spatial pattern in which each special complement of certain

numbers of R_α -groups, R_β -groups, . . . R_ω -groups are inserted in the C_α atoms in the shell, constitutes the specific personality of the individual protein (fig. 1).

SIGNIFICANT ASPECTS OF THE BEHAVIOR OF NATIVE PROTEIN

Many important characteristics of the native proteins have been discovered by Svedberg's work (Svedberg and Pedersen, 1940) and by the other investigations already mentioned. Perhaps the most important for our present purpose are the characteristic ways in which native proteins exhibit association and dissociation reactions under many different stimuli. Thus native proteins, while maintaining their own molecular integrity, exhibit such reactions when the pH of the solution is changed, when the salt concentration is altered, and also when other proteins or molecules of normal size such as amino acids, or acid amides, etc., are added to the solution. Such behavior must of course be specially significant in any consideration of cytoplasmic structure, in view of its composition. In cases of association, the linkings between proteins may not always be direct interlinkings of R-groups. Sometimes they may be made by means of carbohydrates, sometimes by phosphatides, sometimes by inorganic ions, and so on.

Among a welter of experimental facts of these types, the one fact crucial for our enquiry should perhaps be specifically mentioned again. In all these dissociation and association phenomena, there is no evidence that the intimate domestic architecture of the individual native proteins is disturbed. The picture forced upon us is that of the native proteins maintaining their own integrity and, with this integrity maintained, being able to make and break links with other proteins and with foreign molecules.

The effect of dilution on protein particles should also be mentioned. Thus a sufficient dilution of a solution of horse hemoglobin leads to a break-down of the particles into smaller units of about half the molecular weight. Inorganic ions, such as calcium and magnesium, affect protein molecular weights in many cases (Eriksson-Quensel and Svedberg, 1936). These important results, among many others, have led to the picture of some, perhaps all the heavier native proteins, as *molecular colonies*, in which the protein particle, stable under certain conditions only, consists of two or more protein units in association, interlinked directly or by means of metallic ions or other foreign molecules. Thus we have from the outset reason to think in terms of colonies of native units (Wrinch, 1938), a view to which expression has recently been given by Pedersen also (1939).

It is of particular importance for our problem to notice the extent to which, in the case of certain protein particles, the percentage of water determines whether the "molecular colonies" are dissociated or not. Thus when the protein concentration is less

than 0.8 percent, horse CO-hemoglobin particles (Svedberg and Pedersen, 1940) dissociate into halves. For a concentration of 1.0 percent or more, there is little or no dissociation (Svedberg and Fahreus, 1926; Svedberg and Nichols, 1926). In the case of thyroglobulin (Heidelberger and Pedersen, 1935) there is much dissociation into smaller units when the concentration is less than 0.5 percent, but little above this concentration. Caseinogen particles similarly dissociate considerably for concentrations of less than 0.67 percent.

The case of casein is particularly interesting in that the protein units can evidently associate into particles, using calcium ions as a cementing material. Thus Philpot and Philpot (1939) found that casein particles—or molecular colonies—of increasing weights are found in solutions with increasing calcium concentrations.

Other cases for which it seems to be necessary to postulate that the native protein particles are colonies of units include the oil seed globulins (Sjögren and Szychalski, 1930; Svedberg and Sjögren, 1930). Thus the cocosin particle appears to contain an even number of units. Amandin and excelsin particles appear to contain 8 or a multiple of 8 units, and edestin particles 12 units or a multiple thereof. In a very neat experiment the particles of horse-serum albumin, of molecular weight about 67,000, were broken down homogeneously into quarters and subsequently homogeneously into eights by digestion by pepsin for periods of 5 and 30 minutes. Since even at the stage of division into eight parts there is still evidence of affinity between these products and the antibody, it seems possible that the intact particle is a colony structure (Holiday, 1939a).

The case of the hemocyanins, the copper-containing respiratory pigments, is perhaps still more striking. Here we have evidence for a colony of some even number of units for the Malacostraca and for the Gastropoda (Eriksson-Quensel and Svedberg, 1936) a colony of some multiple of 12 units. In the case of the erythrocrurins (Svedberg and Eriksson-Quensel, 1934), the hemin-containing respiratory proteins of the invertebrates, Svedberg has suggested that the particle weights are in many cases some simple multiple (1, 2, 4, 8, 16, 24, 48, 96, 192) of a unit containing one hemin group, a suggestion finding its most obvious interpretation in terms of a colony type of structure.

That such associations are not always between members of the same molecular species is seen from the studies on mixtures of lactoglobulin with serum albumin (Pedersen, 1936). Such studies tend to confirm the conclusion, already suggested in immunological investigations of antigen-antibody complexes, that the association of native proteins depends in many cases upon highly specific affinities between molecular surfaces, for cases are also found in which mixtures of different proteins (e.g. mixtures of serum albumin and some pathological globulins), show no interaction (Pedersen, 1938). In many

cases it was found that the salt concentration and pH medium play little, if any, part; the nature of the individual proteins was the all-important factor. Very striking in this connection are the experiments with serum albumin and helix hemocyanin, in which it was found that the protein molecular weights were affected by the introduction of arginine and ammonium chloride in the first case but not the second and by the introduction of lysine and ammonium chloride drastically in the second case and slightly in the first.

These definite deductions regarding specific association between individual native proteins and between native proteins and foreign molecules, which have thus been demonstrated in several independent fields, allow us to add one further feature to the picture of the native protein units which has been forced upon us. This rigid shell of the protein unit would seem to have sites or patches on it which form faces having a certain spatial relation to one another. I may refer, in particular, to experiments in which serum globulin is used as antigen (Marrack and Smith, 1931). Here it is found that in mixtures with antibody in the proportions which flocculate most rapidly, one molecule of globulin antigen combines with about four antibody molecules. Picturing such associations in terms of the protein unit with its rigid shell carrying R-groups arranged in a definite pattern, we seem forced to think of the essential determinant groups on the antigen protein as occurring on different areas or faces of the shell, thus facilitating the grouping of as many as four antibody proteins around it. Indeed it seems convenient to think of the outer surface as in the form of a polyhedron, with definite patterns, which in many cases are immunologically significant, repeated on a number of faces held in definite orientations to one another, since this makes the picture still more definite. These patterns on the polyhedral surfaces are, we must presume, extremely "sticky" in the sense that the atoms on the faces readily form associations with certain atoms on certain other faces and such associations in many cases are specific, implying that R-groups on both surfaces are involved in the association. That the shell is also capable of non-specific association with simpler molecules such as water has already been mentioned. In these cases the association no doubt depends, in part at least, upon the skeletal atoms of the shell, namely upon the oxygen, hydrogen and nitrogen belonging to the skeletons of the constituent amino and imino acid residues.

The results of drying native proteins are also to be specially noticed, for they tend to show that native proteins cannot exist as isolated units unless water, or other molecules or ions, capable of interlinking with them, are present and linked to them in a definite manner. Thus it has been found that the addition of sugar, NaCl, alanine, glycine or gelatin to native proteins before drying protects them in their native state to some extent, and that most

proteins cannot be dried in the absence of some protective substance and yet maintain their native structure intact (Brosteaux and Eriksson-Quensel, 1935). Also significant is the fact that "it seems almost to be a rule that measurable interaction between proteins occurs at a much lower concentration when they are 'purified' than when they are in their native condition and environment" (Svedberg and Pedersen, 1939). This finding, which is based upon a wealth of experiments, would seem to make it specially clear that native proteins tend always to form interlinks with other molecules or ions; that, in fact, their surfaces are extremely "sticky." This aspect of the native proteins alone will prove of the greatest significance in relation to the present problem. As an indication of the great variety of molecules which have been found to stick to native proteins, we may refer to the work of Przylecki (1939) which is concerned with combinations of native proteins with nucleic acid, protamines, phosphatides, polysaccharides, fatty acids or lipins, sterols or carotenoids. The many cases in which carbohydrates in particular are found in association with native proteins (e.g. the carbohydrate-rich fraction of serum albumin, Kekwick, 1938) deserve specific consideration.

The many virus proteins containing nucleic acid are also, in all probability, directly relevant to our problem. For it appears from recent spectroscopic studies that there is an interplay between the nucleic acid metabolism of the nucleus and that of the cytoplasm (Caspersson and Schultz, 1938). The presence of nucleotides in rapidly dividing tissues may indeed be a general phenomenon (Caspersson and Schultz, 1939). In view of the possibility that the synthesis of some or all native proteins may be dependent upon the presence of nucleic acid or nucleotides, associations of native proteins with such substances may prove of the first importance for cytoplasmic functions and, if so, are certainly of the first importance in cytoplasmic structures.

The full history of the understanding of interactions and specially of associations between proteins and proteins and between proteins and other molecular types is still in the making. For this reason it is hardly possible at this time to give a systematic and logical presentation of the situation and we must content ourselves with pointing to the many facts which throw light on the native protein as it exists and functions in living systems. But these facts at least give some clues to the kind of behavior which we must expect to find above all in cytoplasm where, if anywhere, the full subtlety and intricacy of behavior of which native proteins are capable may be expected to manifest itself.

But at this point we encounter a crucial phase of our enquiry. This architecture, we are told again and again by many workers in the biological field, must be maintained intact if biological functioning is to continue. Thus an investigator whose life has been spent in cultivating an intimate acquaintance with

cytoplasm itself warns us that "chemical analyses do not reveal the original substances in protoplasm, or at least not the original state of those substances. The organization of a protein in one environment is liable to differ from that in another" (Seifriz, 1939). Experiments in which horse-serum globulin was spread as a film on the surface of water into which an antibody to this protein was introduced give precision to this idea, for no evidence of combination between the antibody and the film formed from the antigen was found. It is inferred that the combining groups of the antibody and determinant groups of the protein antigen are not small prosthetic groups but specific patterns of certain amino acids, which, in this case, are disarranged when the protein forms a film (Marrack, 1939). This result certainly shows that even so mild a treatment as the formation of a film can sometimes change the native structure of this protein in a physiologically significant way.

Again from the pen of a leading authority on the proteins, particularly in their biological aspects, we read "The proteins are essential constituents of the body," but, we are warned, "the biological importance of the albumins and globulins in cells appears to lie in their extreme sensitiveness to physical conditions. The movement of water into and out of cells is probably regulated largely by the response of the cell proteins to changes in reaction and salt content. The outstanding character of albumins and globulins is that they can be coagulated by heat. . . . The death of cells also is accompanied by a coagulation of the proteins and it may safely be assumed that life in the cell is associated with the maintenance of the uncoagulated condition of the proteins" (Jordan Lloyd, 1938).

This statement, which is fully warranted, is plain enough. It means that any theory of cytoplasmic structure must allow the native protein units to maintain their individual structure intact. Maybe, in our present unfortunate state of ignorance, we have a great deal of latitude in arranging such units but we have no latitude at all in postulating changes in the domestic architecture of the individual units. The results already cited thus may justify us in postulating changes in relative position of this and that native protein unit. Let us, by all means, consider the possibility that such units may be linked, now to each other directly, now by means of various intermediates and so on. But, in my opinion, it can only lead to error if we allow ourselves to think of individual native protein units as changing the arrangements of the skeletons of their constituent residues. Such disturbances can hardly leave specificities unchanged. At the very least, it seems fair to say that the onus of proof of justification for making such postulates lies on those who venture to take such liberties with native protein units. Thus the "adjustability" of cytoplasm, shown unmistakably in its elasticity and plasticity and structural viscosity can better be accounted for in

terms of differences in the arrangement of the native protein units and other constituents of cytoplasm, than in terms of change in structure within the protein units themselves.

It seems necessary to state this point of view in unequivocal terms for this reason. It may have been natural ten or fifteen years ago to think in terms of the "classical" ways of accounting for the microscopic phenomena as uncoiling chains and "periodic contractions of polypeptide chains." At that time, little or no attention had been given to ancient but vital crystallographic work on a number of proteins (Schimper, 1881) (in which, it might be maintained, the modern picture of the native proteins was already implicit) and it might perhaps be argued that apart from this work there was little indication of the molecular status of the native proteins. However, the last decade has seen a wealth of indications, from many different sources, that the native protein is the key to life processes, that it is the key in virtue of its structure and that its structure is very easily disrupted even by mild treatments. Therefore it would seem of the utmost importance in building theories of cytoplasmic structure to make the fullest use of knowledge of the structure of native protein units obtained by the most delicate methods of enquiry now available. For, it should be emphasized, ultracentrifugal, crystallographic and immunological studies seem to be methods of enquiry which are capable of yielding information about the *intact structures* of native proteins in many cases. Thus in his studies on the phycoerythrins and the phycocyanins, Svedberg specifically mentions that they exist as particles of the same size in the aqueous extract of the algae as in the purified condition. "It is therefore likely," he concludes, "that they appear in the same state in the living cell" (Svedberg and Pedersen, 1940). Actually in such cases where the pH is kept within carefully defined limits, and salt concentrations, etc. are closely regulated, the type of change of structure for which a watch must be kept is, in general, simply an association or dissociation, which is often found to be reversible. Such a change presumably leaves the native structure intact. But in a great many cases there is definite mention of the conditions it is necessary to observe to prevent "the irreversible splitting of the molecule" or the formation of "low molecular weight substances." Thus in the case of tobacco mosaic protein, fifteen hours at a pH of 1.8 leads to complete unhomogeneity (whereas there is complete homogeneity, indicating intact native structures, from pH 2.8 to 5). In the case of tobacco ring-spot protein, one hour at a pH of 3 accomplishes the same result (Svedberg and Pedersen, 1940).

The possibility that native proteins may change their structure is, of course, always in mind in X-ray crystallographic investigations also. Thus in the work on "dry" insulin crystals we are told that "the main part of the sample was used for a test of the

biological activity of the preparation which was found to be 24 international units per mg. This shows that these crystals are biologically active insulin (Crowfoot, 1938). The X-ray pictures obtained for these "dry" insulin crystals record a very limited series of reflections—only 59 terms in all—and no reflections were observed from planes with a spacing smaller than 7.05 Å, so that it seems probable that the close packing of insulin units results in irregularities in the orientations of the various atomic groups in the R-side chains. In spite of this, the native structures of the molecules, it appears, are maintained intact. In the later work on "wet" insulin crystals, thousands of reflections down to interplanar spacings of 2.4 Å are obtained, which indicates regularity in the structure down to atomic dimensions.

The contrast between the degree of perfection of the X-ray photographs of wet insulin, and other "wet" proteins (Crowfoot, Bernal et al., 1938) and those obtained for keratin, silk and other fibrous proteins, reinforces the warning we have referred to above. Indeed it seems very probable that information derived from such inert proteins will prove only misleading when used as a guide in an attempt to understand the nature of protein systems within actively functioning cells—very much more misleading, in fact, than e.g. using information about graphite as a guide to the structure of diamond. Yet there has been an immense preoccupation among workers in the cytological field with these fibrous structures. On some occasions the rich mine of information implicit in the Svedberg studies and in the crystallography of the globular proteins is brushed aside (Seifriz, 1938); in other cases it is not even mentioned. The focussing of attention on the structures which have been proposed for such inert proteins as hair, wool, horn and feather is indeed the less understandable when we realize that at the turn of the century there was in the minds of many a picture of the proteins as "idiosomes" (Whitman, 1893), or "biogens" (Verworn, 1903), in terms of which the activities of the cells must ultimately prove to be capable of formulation. This may not be completely adequate as a viewpoint today, but at least it would seem to show the complete inadequacy of the "classical" point of view.

THE COMPOSITION OF CYTOPLASM AND THE SIGNIFICANCE OF THE PROTEIN COMPONENT

A vivid picture of the curious, varied and indeed unique, properties of cytoplasm has been given by a number of writers. Instead of giving a catalogue of its characteristics, let us begin (as it has been the tradition that enquiries into simpler systems should begin) with a consideration of its composition. Cytoplasm, we are told, contains first and foremost a huge complement of water. A reasonable estimate for the cytoplasm in an actively dividing cell would lie between say 60 and 95 percent. The non-water

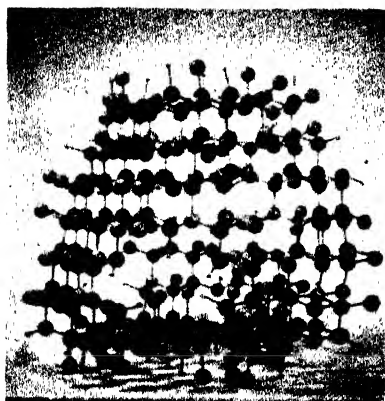


FIG. 1. The Native Protein, pictured as consisting of a rigid multiply connected atomic framework carrying R-groups rooted in a definite spatial pattern of C_α atoms.

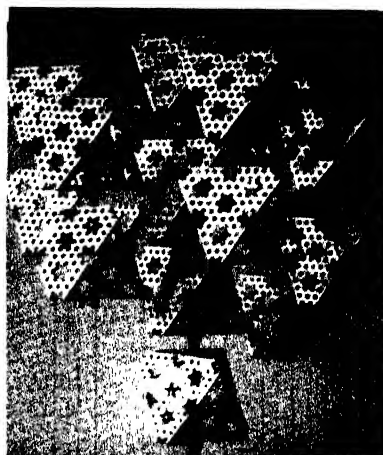


FIG. 2. The crystal lattice of "dry" insulin showing the spatial interarrangement of the protein units according to the X-ray analysis.

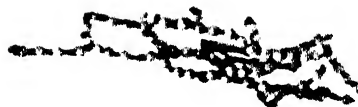


FIG. 3. An example of the type of structure suggested for cytoplasm in which the rigid protein units are flexibly interlinked. Two positions are shown, to indicate the flexibility of the system.

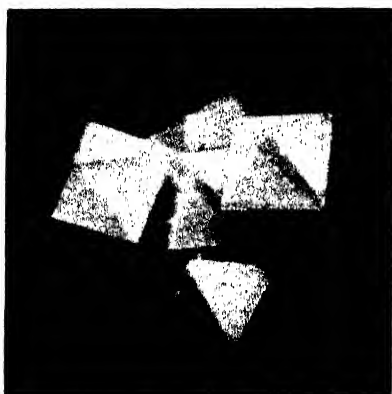


FIG. 4. A 4-way branch point in such a structure in which a single rigid protein unit is flexibly interlinked with 4 other such units.

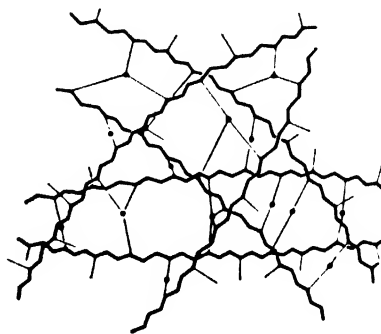


FIG. 5. Structure of cytoplasm after Frey Wyssling (J. Roy. Micro. Soc. 60:128, 1940).

components include proteins, fats, carbohydrates, phosphatides and salts.

For various reasons, it seems proper to give our attention first to the proteins. While it might be difficult to substantiate the view that the proteins are the determining units in the structure of cytoplasm point by point, the general agreement that this is the case makes this the obvious line of attack on the problem. Already in 1878 a philosopher summed up the point of view which should, I think, still be our guiding principle, in these words: "Life is the mode of existence of protein substances." A rather more detailed statement of this same point of view is given by Pauli, who in 1922 said "It would be superfluous to discuss which of the constituents of the living cell are most important in vital processes. Proteins, lipoids and certain inorganic salts are alike indispensable and have a very intimate relation, both physical and chemical, to one another. There is, however, no doubt as to the central position of the proteins in the organization of living matter. Apparently they occur in nature in close connection with vital processes; in the living cell they are completely irreplaceable; and above all, they alone display the specific properties of living matter" (Pauli, 1922).

That these specificities of native proteins must indeed be regarded as the natural basis for the classifications of species and indeed as the basis of the differing personalities of living individuals—that in fact, "the specific character of every animal and every plant may be determined ultimately by the specific characters of their structure-forming proteins" (Lillie, 1918)—is a corollary of this view. Evidence for this statement is afforded by the studies of blood typing in mammals, by the fact that it is possible to establish a plant's phylogenetic position solely by an analysis of its proteins, and by Svedberg's contributions to Comparative Biochemistry in his studies of the respiratory pigments of mammals, birds and fishes. How definitely his studies have caused him to adopt this point of view is shown by his remark that "an investigation of the pH-stability diagram or a determination of the electrophoretic mobility curve together with the normal sedimentation constant of the protein in the blood is sufficient to decide to which species a given hemocyanin belongs" (Svedberg and Pedersen, 1940).

The necessity of attributing this extreme degree of specificity to native proteins, which after all are all composed of the same quite restricted set of building units, gives a further emphasis (if such be needed) to our central point that the cytoplasmic proteins, like other native proteins, form molecular species each with its own perfectly well defined atomic architecture.

Taking then the native protein as the essential structural constituent in cytoplasm, the first question we have to ask is this: what types of structure can be expected of a system containing some small percentage of native protein molecules together with

a very large percentage of water and small amounts of other constituents including carbohydrates, fats and salts?

THE STRUCTURES OF CRYSTALS OF NATIVE PROTEINS

To answer this question, it is natural to turn first to the most precise information available regarding structural systems containing native proteins and water, namely that obtained from protein crystallography. Insulin crystals for many reasons provide a convenient starting point. Two types "dry" (Crowfoot, 1938) and "wet" (Crowfoot and Riley, 1939) (both containing some water) have been studied. In both the molecules are arranged in hexagonally placed parallel columns, unit being stacked squarely upon unit in rectilinear array, with a repeat distance of 30.9 Å for "dry" crystals (fig. 2) and 34 Å for "wet." In both cases the crystal is made up of parallel columns so arranged that the molecules in one alternate trio of the columns surrounding a given column lie a little above and those in the other alternate trio lie a little below the molecules in the central column. Thus each molecule has three nearest neighbors in neighboring columns a little above (actually 10.3 Å and 11.3 Å respectively above) and three nearest neighbors the same distance below, the distance between each molecule and these six neighbors being 44.4 Å in the dry and 49.4 Å in the wet crystals.

The determination of the density allows the cell molecular weight to be calculated in each case. This is found to be 39,700 in the first case and 52,400 in the second. In the first case, it is known that there is at least 5.35 percent of water, so that the molecular weight of the anhydrous insulin molecule comes out at 37,600 at most. This allows us to deduce that in the "wet" insulin crystals there is at least 28.3 percent water.

In studying possibilities regarding cytoplasmic structures we are of course looking for systems containing vastly more water than these crystals, but nevertheless the protein systems in these insulin crystals give a powerful lead. For they show, in the first place, that native proteins can and do arrange themselves in definite orientations to one another; secondly, they give some suggestions as to types of ways in which this is done. Insulin crystals are of special significance for cytoplasmic structure in that the possibility that intact protein units readily form linear sequences—or "fibrils"—is suggested by the existence of the columns of molecules both in "wet" and "dry" crystals. In a crystal large enough to use for X-ray studies such "fibrils" may have length of the order of millimeters or longer. Of course in these cases, the fibrils do not exist in isolation, but only linked together in parallel, forming hexagonal arrays. The persistence of the columns in both the drier and wetter crystals in any case shows that the native insulin units are so designed as to permit the

formation of such linear arrays. This example is concerned actually with a rectilinear array of protein units. We could also select for consideration linear arrays in the form of spirals, in terms of which the insulin crystal can also be described.

Since we are dealing here with definite crystal arrangements of molecules which have molecular weights running into tens of thousands, instead of tens or a few hundreds as in ordinary crystals, it would seem that the stability of the crystal requires us to postulate simultaneous interlinking of the molecules at several points to form the perfectly arranged columns, and simultaneous interlinking at several points between inter-column neighbors. Here again, then, is an indication of some rigid skeletal structure for the molecules. The existence of the columns in both cases is specially interesting. Since such columns apparently do not occur in all protein crystals, the interlinking within columns must involve R-groups whose nature and spatial arrangements are specific for this special pair of areas on the insulin molecule. Now according to our picture the roots of the R-groups of native protein units are arranged in specific positions on the surface of the molecules, such long R-groups as those of say lysine ($-R = -CH_2 - CH_2 - CH_2 - CH_2 - NH_3^+$), and glutamic acid residues ($-R = -CH_2 - CH_2 - COO^-$), being free to curl up. The fact that the intracolumn repeat is 30.9 Å for dry crystals and 3.4 Å greater for wet crystals, then seems to fit nicely with more fully extended R-groups in the wet case and less fully extended R-groups in the dry crystals. Incidentally, it has become customary to assume that when amino acid residues are interlinked, the distance between the roots of the R-groups is of the order of 10 Å. Thus in this case, we have an estimate of the width of the shells of the molecules along the columns of say $34 \text{ Å} - 10 \text{ Å} = 24 \text{ Å}$. From this sketch of the results obtained from the study of actual crystals, it will easily be seen how readily the arrangement of the insulin molecules may be interpreted in terms of the picture of native protein units as polyhedra carrying R-groups. The columns suggest the molecule has a pair of parallel surfaces, since in this way the perfect alignment of these large and heavy molecules into columns, reaching lengths of the order of millimeters, is explained. The three pairs of other closest neighbors suggest three other pairs of parallel faces. Evidently a polyhedral surface, with four pairs of parallel faces, would give a ready interpretation of these arrangements of molecules, both in wet and dry crystals (Wrinch, 1937 a and b). As the simplest polyhedron fulfilling the conditions we may think in terms of a regular octahedron with a distance of 24 Å between parallel faces consequently an edge length of 29.4 Å (Wrinch and Langmuir, 1938).

Two other aspects of these proteins present themselves which are directly relevant to cytoplasmic problems. In the dry insulin crystals under discussion, there is a content of zinc which corresponds

to about three ions per insulin molecule. The closer interlinking of the molecules in columns and the wider interlinkings of inter column molecules, which amount to six per molecule, made it reasonable to suggest that the zincs function as rivets between inter column neighbors. Certainly this apparent stoichiometry would seem to be of some significance and the marked stability of insulin by contrast with other protein crystals would thus find a simple interpretation. It is also relevant to recall the fact that cadmium and nickel and cobaltous ions (but so far, I believe, no others) also facilitate crystallization, the amounts of metal found in certain crystals obtained (.77, .41, and .44 percent, to be compared with .52 percent of zinc) corresponding to about 3 ions per insulin molecule (Scott, 1939). The fact that crystallization proved impossible in the absence of certain inorganic ions, supports this picture of these metallic ions playing a definite part in holding the protein units together, a suggestion which has already presented itself. For our present purposes such a situation has its importance. It indicates that certain inorganic ions in cytoplasm may be used in the same way, so that the continuity of the protein phase may actually be conditioned by the presence of inorganic ions of suitable kinds and in appropriate concentrations. Possibly this suggestion may throw light on the difficult question of ion equilibrium in that the movement of ions in and out of cells may perhaps be conditioned by questions of supply of and demand for rivets of specific design needed for the stability of protein colonies or of frameworks of protein units. The fact that the concentration of calcium in the blood cannot be greater than, say, 15 mg. per 100 cc. or less than 8 mg. per 100 cc. without serious consequences may prove interpretable on the same lines.

SOLUBILITY STUDIES OF INSULIN

The other point deals with some information regarding the arrangements or at least the nature of certain R-groups in insulin. The insulin molecule is known to have about one third of all its residues leucine residues, in which the R-group is $-CH_2 - CH(CH_3)_2$, and so is highly hydrophobic in character. Whether, when insulin is in solution in water, this large number of hydrophobic R-groups is packed away in the interior of the molecule, we do not know. That they may, even under these circumstances, be external to the polyhedral surface is suggested by recent studies, in which it was found that the solubility of insulin in propyleneglycol, $CH_3 - CH(OH) - CH_2 - OH$ is 1.9 grams per liter, to be contrasted with a solubility of less than 0.027 grams per liter in water (Cohn et al., 1939). This fifty-fold greater solubility in the solvent with a hydrophobic group as well as hydrophilic groups, than in water with hydrophilic groups only, may indicate that the leucine R-groups are arranged on the surface of the molecules. If they are so arranged that they can form compact hydrocarbon neighbor-

hoods, after the manner of acetamide crystals, (Senti and Harker, 1940) the appreciable solubility in water can be explained. It would then be natural to suppose that in propylene glycol solutions these hydrophobic R-groups would extend themselves further from the polyhedral surface. A packing with the hydrophobic tails of the solvent molecules in contact with these hydrophobic R-groups and simultaneously the hydrophilic bodies of the solvent molecules in contact with hydrophilic groups (of the skeleton and suitable R-groups) would then account for the higher solubility.

These solubility data bear directly upon the cytoplasm problem in several ways. Just as the solution of insulin in water shows the interlinking of this protein molecule with water molecules, so we expect cytoplasmic native proteins to interlink with carbohydrates known to be present, as well as with water. Similarly, links with the hydrophilic groups of phosphatides known to be present may also be expected. The hydrophobic groups of the phosphatides can be visualized after the manner described for insulin in solutions of propylene glycol, in close proximity to the hydrophobic R-groups of the cytoplasmic proteins. In a structure even as dense in proteins as the pepsin crystal (where there may be 50 percent protein to be contrasted with a third or less in cytoplasm) there is evidently ample room and opportunity for carbohydrates, phosphatides and fats replacing water to be arranged in interspaces between the proteins.

THE WATER CONTENT OF PROTEIN CRYSTALS

Now both "dry" and "wet" insulin crystals have a water content which is too low to be applicable directly to structures suitable for cytoplasm in dividing cells. But in several respects they indicate how certain properties of cytoplasm may be explained. In the first place the system is such that the protein component forms a continuous phase—though metallic ions may, strictly speaking, also be involved—in the sense that one may go from any point on an insulin molecule to any point on any other insulin molecule by means of inter-bonded atoms within molecules or by means of interlinkages (perhaps with the help of metallic ions) between R-groups. Further the water component also forms a continuous phase in the sense that one may go from any water molecule in interprotein spaces to any other without encountering a barrier of protein molecules. Thus the system may be regarded as having two interpenetrating phases. Such a system explains several characteristics of cytoplasm, two things in particular. It explains how a system of native protein and water can have a certain rigidity, namely, by means of a continuous phase of protein units which themselves have rigid structures and are interlinked by R-groups having only a certain amount of freedom to change their orientation. It also shows how such a system can allow passage of water soluble molecules or ions.

The interesting change between the protein arrangements in dry (Crowfoot, 1938) and wet (Crowfoot and Riley, 1939) insulin crystals exhibits two other properties relevant to cytoplasmic problems. Apparently a protein-water system can imbibe or expel water by means of changing the details of the interlinking of intact protein units. The imbibition or expulsion of water in the case of insulin amounted, we saw, to 23 percent. This, however, is not a special characteristic of insulin crystals. In all the cases where wet and dry protein crystals have been examined there is evidence of the same phenomenon (Crowfoot, Bernal et al., 1938). Thus wet chymotrypsin crystals have, say, 26 percent more water than the dry crystals.

One final suggestion regarding native protein-water systems may be gleaned from the study of lactoglobulin. This native protein crystallizes to form beautiful crystals of two types, tabular and needle. Both have 8 molecules per unit cell, but the space group is different in the two cases, so that one is forced to deduce that the proteins interlink differently in the two cases. The dimensions however indicate that if, as we may suppose from the molecular weight determinations by the ultra-centrifuge, the molecules do not differ greatly in size or volume from insulin molecules, the molecules are near enough to form interlinks through R-groups. In the case of ribonuclease, a similar situation exists (Fankuchen, private communication). It occurs in two crystalline forms, monoclinic containing two molecules per cell and orthorhombic containing four molecules to the cell. Here then we have two cases in which the same molecules, with their intact native structures, can apparently interlink specifically in two different ways. This fact I regard as an indication (though in embryo) of the nature of the adjustability, plasticity, structural viscosity and capacity of cytoplasm to flow, of which writers so often speak.

The modern picture of native protein units compelled us to reject any attempt to interpret these and other properties of cytoplasm essentially in terms of gross changes in the architecture of individual protein units. Here, to meet the difficulty created by this rejection, is a definite suggestion, from a protein-water system which has actually been observed, of the capacity of native protein molecules to interlink in different though specific ways, thereby showing how the adjustability of cytoplasm may be understood.

PROTEIN-WATER SYSTEMS

It will be agreed that these observed protein-water systems yield many indications as to the interpretation of the behavior and properties of cytoplasm. The mechanism which they suggest for the imbibition and expulsion of water seems, in particular, to be quite fundamental for an understanding of our problem. The proportions of water so far encountered in actually observed systems—50 percent or

less—may well be of the right order of magnitude to make these structures relevant to such cytoplasmic systems as are found in cells which, though part of the living organism, are no longer actively engaged in cell division.

To conceive of protein-water systems in which the proportion of water is far higher seems, however, to present little difficulty. Let us take as our picture an arrangement of units similar to that in the insulin crystal but slightly modified for the sake of simplicity. We will leave the columns intact, but modify their interarrangement so that the eight neighbors of any unit lie on two "mirror" pairs of tetrahedrally related directions. This means that there will still be six hexagonally arranged parallel columns around any one column, but the parallel octahedral faces on interlinked units will now be centrally apposed, and the distance between the centers of every pair of interlinked units will be the same, say, $d = 34 \text{ \AA}$ as in the case of the units in the columns of the insulin crystal. The units now in fact lie on two interpenetrating diamond lattices, with metric $d = 34 \text{ \AA}$.

Now let us study the results of taking this 8 coordination system, deleting certain units and replacing them by water molecules, and aim at getting systems in which there are very much higher proportions of water than in actual protein crystals. This is easily achieved in the following manner (fig. 3). (It should be emphasized that this is but one way among countless others, since this arrangement of molecules can be described in terms of linear arrays of a great variety of kind, including spirals etc.) Starting at any point of the crystal, retain n units in a string along a column. Now at each end of this string, retain a similar string in each of three other directions which, with the direction of the original string, comprise four tetrahedrally related directions. Continue this procedure indefinitely, discarding all units except those in the strings. Now such a system may be described very simply in terms of a diamond lattice. It consists first of a number of protein units which we may call P_4 units (fig. 4), which have four neighbors and form the (four-way) branch points. Otherwise it consists wholly of the protein units in the strings which are linked to two others only and may be called P_2 units. For every P_4 unit there is a half share of four strings of $(n-1) P_2$ units, that is $2(n-1) P_2$ units. The P_4 units lie on a diamond lattice of metric nd and it is therefore a simple matter to work out the water content of this skeletonized crystal.

Just as a diamond lattice of metric d gives a volume of $V = 8\sqrt{3}d^3/9$ per carbon atom, so our crystal gives a volume of n^3V corresponding to one P_4 unit, $2(n-1) P_2$ units and an appropriate share of the water molecules completing the system. To obtain an estimate of the percentage of weight of water present, we have to adopt some values for W_p and V_p , the weight and volume of each protein unit. The figures for dry and wet insulin crystals

indicate that the volume of each insulin molecule is probably not greater than $47,200\text{\AA}^3$ (a generous estimate) and its weight has been calculated to be 37,600. We adopt these values. It then follows that, of the unit volume n^3V , that occupied by the water molecules is at least $n^3V - (2n - 1)V_p$. Since water will not occupy a volume of more than 30\AA^3 per molecule, its weight must be at least $18(n^3V - (2n - 1)V_p)/30$. This is to be compared with the weight of protein $(2n - 1)W_p$. Thus a conservative estimate for the (weight of water)/(weight of protein) in the system is $18n^3V/30(2n - 1)W_p - 18V_p/30W_p$.

When $n = 2, 3, 4, 5, 6$ this ratio takes the values 2.6, 5.2, 8.8, 13.4, 19.0 so that the percentage by weight of water comes out to be 71.2, 83.9, 89.8, 93.1 and 94.7 respectively. When n is 10 or more the percentage is greater than 98.0; when n is 20 or more, it is greater than 99.2. Evidently the fact that the weight of water is a cubic function of n , while the weight of protein is a linear function of n , implies that the percentage of water can be made as near 100 as we wish, simply by taking n sufficiently large. Thus long enough strings of P_2 units, joined by P_4 branch units, will give an arbitrarily high percentage of water.

These systems comprise a single continuous protein phase and an interpenetrating continuous water phase. While it is convenient to talk of the protein phase as a protein framework, it is however a framework of a special kind. Each protein unit is taken to be a rigid shell, with the atoms of all the skeletons of the constituent residues, $N - C_\alpha(C_\beta -) - C - O$, in fixed spatial interrelationships. But the R-groups have some freedom to turn and twist. Thus a pair of linked protein units cannot recede more than a fixed distance from one another, nor can they approach too near together. Within the restrictions imposed by the R-groups they would however be free to change their relative positions and orientations. In so far as the linear sets of P_2 units are restricted in these various ways, they may be regarded as constituting rods having some measure of elasticity and pliability and also as strings having some degree of rigidity and tensile strength.

Thus, the protein framework, which for simplicity we have taken as arranged in space with its branch units on a diamond lattice, will no doubt be capable of considerable and immensely complicated though definitely restricted contortions, at the cost only of changes in the distribution in space of the constant water complement. Even at this stage, therefore, it is useful to consider our structure in direct relation to the properties of cytoplasm. First and foremost, it may be suggested that the fact that, in a certain sense cytoplasm has been regarded as a solid (though an anomalous one) and, in a certain sense, as a liquid (though an anomalous one) may be simply interpreted in terms of a protein framework, with an immense interpenetrating water phase. For the water molecules can flow and function (in cer-

tain respects) as a liquid in virtue of the large volumes they occupy in framework interspaces and in virtue of the measure of freedom to turn and twist possessed by the units of the protein framework. The protein framework functions (in a sense) as a solid since this measure of freedom is a greatly restricted one, giving the framework some measure of rigidity and some tensile strength.

Such a water-protein framework system will also show *plasticity* if a sufficient force is appropriately applied, since protein units sticking together may be torn apart and form new links with other protein units. The many data cited earlier, showing how mild treatments (even dilution) cause protein units to become disengaged, suggest that such a force need not be large. The fact that microscopically visible particles do fall through cytoplasm incidentally suggests that the protein framework contains strings of considerable length, or that branch point or other units break away from their neighbors very easily as just suggested. A framework with a distance of say 1,000 Å (about 30C₂ units) between the branch points might be required to allow passage of such a particle without rupture of the framework.

An independent suggestion that protein units in the framework are torn apart even by small forces, is, however, contained in another well known fact about cytoplasm (Seifriz, 1939). Thus the viscosity of cytoplasm depends upon the force with which it is pressed through a capillary, and protoplasm will of itself pass through parchment paper with pores 5×10^{-5} mm. in diameter, but if pressed through the much larger pores of silk, 5×10^{-2} mm. in diameter, it will not survive. Apparently the pressure applied breaks the framework drastically, perhaps so drastically that the native structures of some of the proteins are destroyed.

The types of protein framework devised indeed appear to offer an interpretation of the property of "structural viscosity" indicated by the following experiment (Scarth, 1927). When the fall of small particles of gypsum through the cell sap of the vacuole of the alga *Closterium* is observed, a well defined viscosity, 2.5 times that of water, is found by applying Stokes' law. "If we repeat this experiment by observing the fall of small particles through the cytoplasm, we shall not get a satisfactory result, because there is no constant velocity of fall." "Sometimes," the writer adds, "the movement of the particles seems to be hindered in the same way as the fall of microscopic particles through a heap of brushwood." A protein framework-water system such as we have devised might well be expected to yield such results.

So far we have considered the behavior of the systems under study when the complement of water is that required to keep all the protein units at their full distance from one another. But there seems every reason to anticipate that such a system can continue to exist—without damage to the atomic

architecture of the protein units—even when considerable amounts of the water are withdrawn, and later restored. Thus the system throws light on the swelling and shrinking of cytoplasm by imbibition and dehydration, and in particular on the striking fact that a seed can exist in a comparatively dry state for long periods without impairing its potentialities, its power to function as the repository of pattern for a complete organism being restored with an adequate uptake of water. For when water is withdrawn, protein units previously far apart, may come near together and stick to one another without damaging their own structures. That the entry of more water molecules will tear them apart again without modifying their native structures is suggested by the results on the dissociation of protein particles into smaller units recorded for hemoglobin and other native proteins already referred to. It is interesting to notice that such considerations offer a simple interpretation of the remarkable fact that a jelly-fish which is "rigid enough when stranded to support the weight of a man without collapsing," though containing 97 percent or more of water, is soon reduced to a thin film if left to dry in the sun (Seifriz, 1938). A system of strings of sufficient numbers of P₂ protein units, interlinked by a small proportion of P₄ units, could show a dehydration even of these striking proportions if it shrinks so that its protein units are about as close together as the insulin molecules in its crystal. Any orderly arrangement such as we find in crystals is, of course, not to be expected under such circumstances. Similarly in the case of developed cells which represent a large amount of dehydration, collapsed protein frameworks would not be expected to show regularities capable of being recorded by means of X-ray analyses (Harrison, Astbury and Rudall, 1940).

So far we have suggested systems in which the complete complement of native proteins is used to build a single continuous quasi-solid phase. But with sufficiently high water complements there is no reason why there should not also be native protein molecules in solution. We have already seen that, in many cases, native protein molecules stay in solution without aggregating, when the protein concentration is not greater than, say, 0.5 percent. Such a concentration means that one protein unit of weight 37,600 and volume 47,200 Å³ must be accompanied by at least $199 \times 37,600/18$ water molecules, taking up say 30 Å³ each, in all a volume of 12.47×10^6 Å³. Thus a volume of 12.52×10^6 Å³ or more could have one free protein molecule replacing a certain amount of water, with protein concentration of less than 0.5 percent. Hence the protein water systems with $n = 20, 30$ which have volumes of water of 484×10^6 Å³, $1,634 \times 10^6$ Å³ associated with one P₄ unit and 38 or 58 P₂ units could have 39 or 136 native protein units in solution respectively. Thus in these two cases 50 and 70 percent of the protein component could be in solution, the strip of P₂ units reaching a length of say .07 and .10

microns. Since the water volume, and therefore the allowable number of free protein units, increases with the cube of n whereas the number of protein units in the continuous phase is proportional to $(2n - 1)$, it is plain that the proportion of the total protein component in solution can be made as big as we please by taking n sufficiently large.

General conclusions thus emerge from the very simple types of protein-water systems so far discussed as follows. By taking sufficiently long strings or chains of P_2 protein units, the percentage of water in the system can be made as large as we please and the ratio of the amount of protein in solution to the amount of protein in the framework can also be made arbitrarily large.

There is thus no difficulty in visualizing protein-water systems consisting of continuous protein phases, and a continuous water phase and native proteins in solution, in which the water content is as high as we please. With the picture of vast numbers of P_2 protein units in strings joined by P_m units ($m > 3$) which constitute the branch points of the system, there are considerable areas of the surfaces of the protein units available as sites for molecules of other types, since only a small proportion of their surfaces are adhering to each other. In the systems so far discussed, all the sticky portions of the surfaces of the protein units are pictured as covered with water molecules, except for those which stick to one another. We can, therefore, easily picture the inclusion in systems of this kind of the various other types of molecules known to be present in cytoplasm.

The carbohydrates and phosphatides can replace certain numbers of water molecules by sticking to the protein surfaces through their hydrophilic groups. The phosphatides, through their hydrophobic groups and the lipids can find convenient anchorages in the hydrocarbon neighborhoods made up of the hydrophobic R-groups of the protein units. In particular, the anchoring of nucleotides on protein units may be expected in view of the large number of nucleoprotein complexes now found in virus researches.

In the protein-water systems any proportion of water, no matter how big, can be obtained by taking strings of P_2 units sufficiently long. Hence, while precise figures for the proportions of the carbohydrates, fats and phosphatides are hard to come by, there is no difficulty in visualizing systems containing these constituents as well as protein and water, with very high water contents.

The simple picture of a single protein framework with attached foreign molecules and proteins in solution in vast water spaces described above, suggests many others. Thus, suppose we start with one unit of the crystal as before and go up along a column and proceed as before and suppose we make another start at a point below half way between two four-way branch points of the original framework and again proceed as before, choosing now the

mirror system of tetrahedral directions. We shall then have two independent interlacing protein frameworks. For a sufficient high percentage of water, many independent protein frameworks can coexist in a given space. With several such protein phases plus the continuous water phase occupying a given region, a most interesting situation results. For two points very far distant may belong to one continuous protein phase, whereas two points much nearer together may not. Thus a torque applied to a P_2 unit of one protein phase may lead to a considerable rotation of another unit of this phase quite remote from itself and yet leave a nearby protein unit belonging to a different phase little affected.

A further suggestion may also be added. We might start with a group of linked protein units and taking this as a super unit proceed as before, going along a column for n such super units and then branching in the three remaining tetrahedral directions as before. Such a framework would have thicker rods. Thus many interlacing protein frameworks made up of rods of various degrees of pliability, rigidity, and tensile strength may also be envisaged.

The type of system here suggested was pictured at the outset in terms of a crystal in which the protein units are arranged in an 8 coordination pattern, topologically identical with those found in the insulin crystals. While, necessarily, specific examples only have been selected for description, a reference to this 8 coordination pattern will enable us to appreciate more adequately the full generality of the structures suggested for consideration.

Thus any crystal with deletions will do, provided certain conditions are satisfied. Let us, therefore, in the first instance delete at will a large proportion of the units in this crystal, replacing them in small part by carbohydrates, fats, phosphatides, etc., appropriately linked to the remaining protein units at hydrophilic groups of their skeletons or R-groups and by juxtaposition to hydrophobic R-groups, the remaining spaces being filled by water molecules. The condition to be satisfied, if the water complement is to exceed say 80 percent, and the protein component is to be say 10 percent, is simply that enough protein units be deleted in the first place, leaving only some small proportion in position and that only a suitably small number of the carbohydrates, phosphates, fats, etc. be inserted in their places.

In the course of deleting this large proportion of protein units, it may so happen that colonies of a few protein units or even single protein units are left isolated. These represent units or particles in solution and the second condition comes in, in that a single isolated protein unit in a sea of water molecules must have a complement of water molecules around it sufficient to reduce the concentration of protein below some appropriate figure (say 0.5 percent). For colonies consisting of two or more units there will be the same type of condition, but

here the concentrations allowed will be somewhat higher. [So far there are only a few data to guide us in the selection of actual values for these concentrations, but we might take the dimeric horse hemoglobin as a guide to concentrations allowed for two-membered colonies (say 1.0 percent) and the other protein particles, for which larger colony structures have been suggested above, as guides in other cases.]

In studying the disposition of the remaining protein units, a third condition is imposed, namely, there must be one or more protein frameworks extending through the whole space. The high percentage of water then makes it necessary that considerable proportions of the proteins in the frameworks should be present in the rods or strands, either as P_2 units forming the most pliable and thinnest strand, or units of higher connexity forming composite columns, that is thicker strands having less pliability. Some P_1 units may also be present. The branch point units will be 3-, 4-, 5-, 6-, 7-, or 8-way units. With high enough water complements, large numbers of interlacing protein frameworks may coexist in the same portion of space.

We have given this picture of an "emaciated" insulin-like crystal, which is more comprehensive than descriptions of any specific system can possibly be, so that the full complexity of the types of systems under consideration may be appreciated. Of course even now we have limited ourselves to systems in which all the protein units are the same size, a limitation which is not a necessary one. Further, there is no reason, even with the octahedral picture of the individual protein, to restrict our consideration to systems in which such protein is linked to, at most, eight other proteins. Interlinking may also be associated with twelve edges of the octahedron, suggesting the possibility of up to 12 neighbors. In any case, a larger protein may attach more than one smaller protein to one face or edge.

In stressing the prodigious complexity of many interlacing frameworks, many attached foreign molecules and proteins in solution in various states of aggregation in the vast water spaces, we wish simply to stock our minds with the many different situations requiring consideration and study, which may arise locally in such structures. But it should be stressed that in proposing that such systems be considered in investigating cytoplasmic structure, I do not suggest that actual cytoplasm is built up on any such random scheme. Cytoplasm from a certain cell of a certain individual no doubt contains perfectly definite proportions of each constituent type of molecule and it "happens" to have just these proportions because they can build themselves into a definite specific structure. "It must be emphasized," writes Frey-Wyssling (1940), "that the cytoplasm is not a 'fluid in motion,' the components of which move in accordance with the law of probability, but that there must be a wonderful architectural arrangement of all the different elements within the living cell." The complexity of the problem and

our present lack of knowledge even of the composition of cytoplasm makes it not yet possible to prove the correctness of such a viewpoint. Nevertheless, I feel convinced that such a viewpoint is the right one to adopt as a guide until the contrary is established. Increasing knowledge derived from studies in structure chemistry tends year by year to substantiate the view that molecular species fit together in well-defined ways. The picture which has been forced upon us by immunology, protein crystallography, and ultracentrifugal and related studies, makes the native protein a well defined molecular genus. As such, it is subject to the rules of association established for simpler molecules. General considerations of great weight make it necessary to assume that the proteins in cytoplasm have their native structures. It follows then that cytoplasm may differ from all the systems so far studied in complexity but differs from them only in complexity. If and when the structure of cytoplasm becomes known in the remote future, I for one expect to find this necessity revealing itself at every point.

CURRENT THEORIES OF CYTOPLASMIC STRUCTURE

It is essential in putting forward a case for the native protein type of structure proposed for cytoplasm in this communication to examine its relation to the theories current at the present time. We may select for special discussion the interesting *reticular* theory which has a long history behind it and has recently been reformulated by Frey-Wyssling (1940).

This writer, discarding the *alveolar* theory, the *granular* theory, and the *fibrillar* theory for various weighty reasons, takes as his guide the two assumptions we have also adopted. The first of these is the assumption which is the basis of structure chemistry in general, namely that the properties of cytoplasm or any other piece of matter should be studied in direct relation to the atomic architecture of its components. The second is the assumption that it is the protein components of cytoplasm whose (atomic) architecture is the dominant factor in cytoplasmic structure.

The structure of cytoplasm, on this theory, consists of loosely interwoven protein strands. Described in these terms it is hardly to be distinguished from the theory we have put forward. To demonstrate this point, we may take Frey-Wyssling's picture of the structure of the cytoplasm (fig. 5) and compare it with the suggested type of structure shown in Figure 3. The respect in which the present theory differs from that of Frey-Wyssling thus relates exclusively to the analysis to be given to the strands in terms of atomic architecture. This fact makes it unnecessary to do more than refer briefly to the way in which such protein-water systems fit and interpret many data relating to cytoplasmic behavior, and the reader is referred to Frey-Wyssling's article for detailed discussion of this point.

Granted that protein frameworks with strands as

their chief feature are required, the interpretations offered, however, diverge fundamentally. Two assumptions determine the course of the analysis by Frey-Wyssling; the assumption that a strand reaching microscopic dimensions must necessarily mean long chains of atoms arranged in parallel, just as a string may be analyzed into a bundle of threads; and the assumption that the only ingredient available in devising protein structures is the long polypeptide thread. I suggest that alternatives to both these assumptions exist and that, in fact, neither is justified in the present state of knowledge. Incidentally it is worth while to call attention to the way in which each assumption has often been used to bolster the other, each thereby gaining a spurious support from the other. Thus, if a microscopic strand is a bundle of threads of atoms, the existence of a protein strand may mean that the proteins are made up of atomic threads; if a protein is made up of atomic threads, the existence of a protein strand may mean that strands in general are bundles of atomic threads.

The body of this communication has already made it sufficiently plain that a linear structure need not consist of atomic chains in parallel. (This point is of the utmost importance in discussing structures for gene proteins (Wrinch, 1940a).) Just as a column in a building consists of bricks arranged in various patterns, so a thread of cytoplasm may consist of globular (or polyhedral) units arranged in various patterns. We have taken actual facts regarding ways in which such units are linked together in the insulin crystal and have shown how, on the basis of these facts, globular units may reasonably be presumed to be capable of forming strands. These strands, we have shown, have properties intermediate between rods with some pliability and strings with some rigidity, as required by the data relating to cytoplasm. The predominance of such strands we interpreted as a necessary characteristic of a structural system in which there is so large a percentage of water as in cytoplasm. There is therefore no justification for the assumption that microscopic strands necessarily mean bundles of atomic chains. The assumption that globular (or polyhedral) units are incapable of accounting for the existence of cytoplasmic strands is generally made implicitly, but one occasion on which it has been explicitly stated throws much light on the lines of argument employed. In a polemical passage, Seifriz (1938) writes: "There has of late crept into the literature information to support spherical protein molecules. It is difficult to do anything structurally with a spherical particle. . . . A spherical protein molecule in solution will mean Newtonian behavior, yet if there is one firmly established property of many protein solutions, it is their anomalous flow. Mechanical properties, such as elasticity and tensile strength, are equally difficult of interpretation. To attempt to satisfy these physical properties with a spherical molecule is rather like asking a

weaver of cloth to make his fabric of sand instead of threads." This colorful passage puts the ideological situation in a nutshell. There would indeed be difficulties in getting structures with elasticity and tensile strength out of a collection of sand particles. But a sand particle is not the modern picture of the native protein. Native proteins in general apparently have very "sticky" surfaces, in the sense that they readily interlink with foreign molecules of a variety of types and above all with water molecules. Further they have on their surfaces patches which have a "specific stickiness," by means of which they can interlink preferentially with other individual proteins and with certain foreign molecules. Finally these patches are apparently spaced on their surfaces so that other proteins are held in position within certain limits. Thus the interlinking of proteins, it appears, puts certain restrictions on interdistances and mutual orientations of the protein units. Replacing Seifriz's sand particle (admittedly incompetent for the purpose in hand) by this picture of the native protein, based upon decades of research in immunology, crystallography, and physical chemistry, there is, as we have shown, no difficulty in building structures with the properties appropriate for cytoplasmic strands or rods. The tobacco mosaic virus protein particle, several thousand Ångströms long and about 150 Å across, may be just such a case, in which a rod-like structure is made up of globular units. For in many different states—crystals, dry gel, wet gel, in solutions (oriented by flow), X-ray patterns have been obtained (Bernal and Fankuchen, 1937), all of which indicate a pattern of some complexity with a repeat unit along the rod and normal to the rod in two hexagonal directions of dimensions $66 \text{ Å} \times 88 \text{ Å} \times 88 \text{ Å}$. Such indications suggest that the particle is a giant colony of large globular protein units. With regard to the second belief I would suggest that there is no evidence at the present day that any cytoplasmic protein is made up of polypeptide chains. In this connection attention may be drawn to the fact that all the evidence adduced as a basis for such a view of protein structure has reference either to such proteins as keratin, or to native proteins whose structures are known to have been altered. It is hardly necessary to argue the unjustifiability of imputing to the cytoplasmic proteins, concerned in the most fundamental vital activities of the organism, atomic architecture which has been proposed for the scleroproteins which have left the metabolic cycle. There is today nothing to show that such a procedure is justified; rather there would seem to be an overwhelming *a priori* improbability that the atomic architectures involved in such different roles in the organism should be of a similar nature. The evidence adduced from studies of maltreated native proteins would appear to be equally clear. The one (perhaps the only) certain thing about these proteins is that they no longer have their intact native structures. I should, therefore,

be of opinion that little regarding the structure of the native protein can be deduced, no more, to use Langmuir's words (1939), than could be deduced about the architecture of Ypres, if you were first to let an army bombard it, and then examine the ruins. If, however, some deduction is insisted upon, it might have to be that the native protein does not consist of polypeptide chains.

Though any deduction about the native protein based on suggestions regarding structures from which it is known to differ must be held suspect, it may be pointed out that this conclusion—that native proteins do not consist of polypeptide chains—is strictly in line with many chemical and physico-chemical facts, and that the case against the polypeptide hypothesis for native proteins has already been argued on many grounds (Langmuir, 1939; Wrinch, 1938c). Even at the very outset of the crystallographic study of native proteins, Bernal and Crowfoot (1934) suggested on the basis of their work on pepsin that "peptide chains in the ordinary sense may exist only in the more highly condensed or fibrous proteins."

THE FABRIC THEORY OF THE STRUCTURE OF NATIVE PROTEINS

It seems pointless to refer to the fact that there are many reasons for distrusting the traditional belief that native proteins are made up of long polypeptide chains and not refer to the attempt which has been made to offer a theory of native protein structure in accord with the facts of crystallography, immunology and physical chemistry; pointless also to construct complicated systems of native protein units for cytoplasmic structures without referring to specific structures which have been proposed for native proteins in general (Wrinch 1934, 1936a, 1937d, 1937e, 1940b, 1941a), especially in view of the fact that such considerations lead to a picture of the native protein which accords with that derived from protein crystallography, immuno-, enzyme- and physical chemistry. The nature of this attempt (which has been frequently misunderstood, in spite of the plainest statements made at our Symposium in 1938 and on other occasions), can be described in simple terms. If a native protein with, say, several hundred residues has a definite structure in which the R-groups alone have some latitude to arrange themselves (to extend themselves more or less fully, etc.) then the atoms of the skeletons of the residues must be built together on some comprehensive plan, in virtue of which they form a definite spatial pattern. A plan of this kind requires that, in some or all cases, each residue be attached to more than two others, which implies that, in most or all cases, the residue cannot play its functional role in the protein in the form of a di-unit — HN — CHR — CO — having connexity of order two. Some or all must be units of higher connexity, say tri- or tetra-units or higher. The moment that residues are allowed to function in this way, a vast

range of patterns of many different types presents itself for consideration. It may be that any adequate discussion of native protein structure should be conducted on the most general lines, but such a procedure in protein chemistry today would evidently defeat its chief purpose namely of stimulating discussion by workers in the protein field of some, or any, of the new types of structure which geometrical ideas immediately suggest. Since there are indications from very many experiments that the native protein has well defined spatial structure, with the roots of some or all its R-groups in a definite pattern on its surface, our attention so far has been devoted to devising ways in which the residues can be woven into surface patterns or fabrics. This is the genesis of the original (lactim) cyclol fabric (Wrinch, 1937d and e), and modified fabrics, the enol cyclol fabrics (Wrinch, 1940b, 1941a) and the various hydrogen bond fabrics (Jordan Lloyd and Wrinch, 1936). All these share the characteristic that most or all the residues function as units of connexity higher than two. These cyclol cages had the interesting property that they accounted for the existence of genuine molecules with weights running into tens of thousands, since each forms a single covalent unit; all suggest from the very beginning how it is that a protein unit may have one or more trigonal axes and one or more digonal axes—characteristics known to be possessed by some proteins, which are completely unintelligible in terms of polypeptide chains. They also accounted for the molecular weight classes of Svedberg in terms of cages with definite complements of residues. There is no need on this occasion to make more than a passing reference to the fact that the cage structures have been shown to fit and indeed to interpret the facts of protein chemistry at many points (Wrinch and Langmuir, 1938; Langmuir, 1939; Langmuir and Wrinch, 1939; Wrinch, 1938d) (including immunochemistry) or to the fact that no contraindication has yet been obtained from studies in organic chemistry (Wrinch, 1939a, 1940c), bond energy calculations and calculations of interatomic distances (Wrinch, 1941b) spectroscopic (Holiday, 1939) or crystallographic (Wrinch, 1939b, 1940d; Langmuir and Wrinch, 1939) studies. But there is a certain methodological and general interest in noticing how neatly the cage structures fit the requirements in this new field. Since the valency requirements of atoms are built essentially on a framework basis, any cage structure must be polyhedral. The way in which the insulin crystal structures (Wrinch and Langmuir, 1938; Wrinch, 1938d) lend themselves to a simple description in terms of polyhedra, such as octahedra (or truncated tetrahedra) thus deserves a word of comment. For it will be remembered that the C_2 structure predicted for molecules in the insulin molecule weight class (Wrinch, 1937b) actually have a skeleton in the form of a truncated tetrahedron which may be replaced, for the sake of simplicity, by the " C_2 octa-

hedron" (Wrinch and Langmuir, 1938). The fact that (as shown above) there seems some reason to deduce from the columns of molecules in the wet insulin crystal an octahedron of height about 24 Å and the fact that the lactim and enol C₂ octahedra are of height 16a, which is equal to about 24 Å (since a, the mean of C—N and C—C distances, is in the neighborhood of 1.5Å), provokes interesting reflections, as do also the relations between these structures and the X-ray data in general (Wrinch and Langmuir, 1938; Wrinch, 1938d).

Whatever the actual facts may be, whether native proteins are cyclol cages or polyhedral structures of other types, it is apparent that the types of structure which polyhedral units can build are able to explain many aspects of cytoplasmic behavior. There is, however, an outstanding characteristic of the cyclol fabrics in particular which fits specially well with the cytoplasm data, just as it fits with many facts discovered by the ultracentrifuge and X-ray analysis. I refer to the high degree of "stickiness" of both types of cyclol fabric.

For these fabrics are extremely "sticky" in that they can form many hydrogen bridges (through their —OH and C=O and =N —or =NH groupings) to other such fabrics or to foreign molecules or ions carrying appropriate atomic groupings (Wrinch, 1937f). Thus it has been calculated that there are sites on cyclol fabrics for hydrogen bridges amounting to three or more per residue (Wrinch, 1937f). It is also worth mention that the enol cyclol fabrics have their NH groups around the lacunae so spaced that inorganic ions of appropriate design may fit into them, after the manner of the nickel and platinum phthalocyanines, (Robertson and Woodward, 1937, 1940) and thereby give additional stability to the fabric. Since each cyclol structure has a definite number of such lacunae, certain types of ion in stoichiometric proportions may be required. Here we have another indication of ways in which concentrations of such ions may be controlled by the proteins.

The cyclol fabrics also have specific patches of stickiness in virtue of particular arrangements of R-groups containing such groupings as —COO[−], —NH₃⁺, —CO—NH₂, —OH, C=O, =NH, ≡N, etc. The presence of so many non-protein molecules such as carbohydrates, phosphatides, etc. in cytoplasm, which presumably fulfill important functions in virtue of their positions in the organized structure, suggests that the native protein surfaces must have a very large capacity to anchor foreign molecules. In this connection, it is worth attention that a completely cyclized lactim residue in the form =N—CHR—C(OH)= has as many sites as the linear form —NH—CHR—CO— while the completely cyclized enol residue in the form —NH— $\overset{|}{\text{C}}\text{R}$ —C(OH)= may actually have more. This is, I think, an important point in favor of cyclol fabrics, as against hydrogen bond fabrics. When a fabric is formed by hydrogen bridges the

residues so far as covalent bonds are concerned, are in the linear form. Since some part of the capacity of the residues to form hydrogen bridges is devoted to the formation of the actual fabric, this fabric will have a smaller capacity per residue than the linear residues themselves, smaller that is to say than a cyclol fabric.

It should also be pointed out how well the picture of hydrogen bridges (Huggins, 1919, 1921, 1922; Latimer and Rodebush, 1920) as the interlinks between some at least of the native protein units in the frameworks fits the requirement that the structure of cytoplasm has considerable plasticity and can be damaged even by mild treatments as mentioned above. For the energy in a hydrogen bridge is very much less than in such covalent bonds as carbon-carbon, carbon-nitrogen and sulphur-sulphur, perhaps ten or fifteen times as small. Thus so many as ten (say) would give a lower energy of interlinkage than a single such covalent bond. Further, many more than ten of them would constitute a linkage more easily broken than one bond, since only some of them would have to be broken at once. If then we postulate interlinkages of this kind at points of the protein framework, we may expect rupture of the framework under treatment which leaves the structure of the individual units intact. It will be noticed that there is nothing in the facts about cytoplasm which makes it necessary that all interlinkages within the protein frameworks should be specially weak and in the case of many interlinkages the possibility of covalent bonds (e.g. CO—NH bonds between acidic and basic R-groups, and S-S bonds as in cystine) should also be considered.

A second type of stickiness, which may supplement those already considered but which alone would lead to very weak interlinks, could be caused by propitious arrangements of R-groups containing hydrocarbon groupings such as —CH₃, —CH₂—, —CH=, etc. Both types of cyclol fabric can provide many such situations. This point has been mentioned in connection with the solubility studies on insulin which uncovered a much higher solubility in propylene glycol than in water. This fact seems to point to the arrangement of leucine residues in such positions on the cyclol fabrics that they can form hydrocarbon nests, which provide convenient anchorage for the propylene glycol tails, but have no affinity to water molecules. In like fashion, we can picture the hydrocarbon tails in fats and phosphatides anchored in definite positions on the surfaces of native proteins in the cytoplasmic frameworks. The merit of fabrics in this connection is plain. Hydrocarbon R-groups of appropriate types fixed onto a framework could hold hydrophobic groups of foreign molecules with a certain firmness which would be lacking if their roots were not held in a definite pattern in space.

It would be a difficult matter to analyze in further detail the many ways in which the fabric theory

of the structure of native proteins dovetails easily and elegantly with this and that phase of cytoplasmic behavior. This new field of application of the fabric theory seems to me to give further support to an answer in the affirmative to the plain question that I posed at our symposium on proteins here, three years ago: "Is there a protein fabric?" I formulated my contribution in terms of this clear and fundamental issue in the hope of enlisting the collaboration of the Symposiasts in obtaining some measure of agreement on this—or any other—basis. Any attempt to force the issue regarding the correctness of specific fabric structures must necessarily fail until a crucial test has been devised. Such a test would therefore be of real importance. The hope, however, proved vain and the importance of the wider issue remains largely unappreciated.

The data on the structure of cytoplasm in the present state of our knowledge also provide no quantitative test for any specific fabric structures. It is for this reason that the present thesis has been developed in terms of a globular or polyhedral particle, the arguments used being independent of any specific atomic pattern, independent even of whether the protein unit is a surface or a volume pattern of the residues. But I think in the course of this development it becomes apparent that a sticky polyhedral cage structure may be capable of playing the rich variety of roles required in living systems, the sum total of which determines the structure of cytoplasm in its most essential aspects. The fact that this picture of the native protein is derived from fields of study quite remote from cytoplasm once again suggests a fundamental unity in all structure problems of protein chemistry.

SUMMARY

Recent work on native proteins, in crystallography, immunology, and physical-chemistry is reviewed and shown to yield a picture of the native protein as consisting of isolated or associated molecular units, having rigid globular structures, on the surface of which some or all the R-groups are rooted in definite spatial patterns. On this basis, a new picture is suggested for the structure of cytoplasm, consisting of interlacing native protein frameworks, to which fats, carbohydrates, water, and other foreign molecules are attached, located in an immense interpenetrating water phase, possibly with proteins in solution. The properties of structural systems of this nature are discussed in a preliminary manner and it is shown that many of the characteristics of cytoplasm prove interpretable in terms of such systems.

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DISCUSSION

DAVENPORT: I am very enthusiastic about this paper. The importance of water is indicated by the fact that vital processes require an abundance of water. In the 1890's, I measured the water content of frog embryos; when laid, the eggs were 50 percent water; in two weeks the amount of water in the developing tadpoles had risen to 94 percent, while the dry substance had slightly fallen off during development. We are too much concerned in genetics with end morphology and too little concerned with the mechanism of development which brings the end morphology about. We underestimate the fact that development depends on increase of substance differential growth—in which water plays an enormous part. The colloidal substances increase also. In the case of bone, the humerus of a fetus adds 500 micra every day at the tip, and during the adolescent growth spurt the boy adds about 100 micra in 24 hours. How important the increase of the vital stuffs is in growth and how little we know about their proliferation; but we do know that in the rapidly growing stage water is supremely important.

MAZIA: It is possible to have cytoplasmic structure without much structural viscosity. Paramoecium cytoplasm, measured by centrifuging starch grains, gives no evidence for structural viscosity.

WRINCH: I think that the type of structure that I put forward for discussion would be capable of fitting in with data of that kind. A low degree of structural viscosity would be associated with the case where individual fibers are fine and long.

WRIGHT: What is your conception of growth? Is there increase in the length of the chains?

WRINCH: I feel that a necessary preliminary to the understanding of growth is an understanding of protein synthesis.

WRIGHT: Is the length of the chain between linkage points definitely determined?

WRINCH: In as far as we have in mind such inter-linkages as (for example) hydrogen bridges between, say, glutamic acid and lysine R-groups, the variation in length could not be very great, say three to five Å.

WRIGHT: Would growth occur on the surface of the molecule?

WRINCH: It may be presumed that the processes of growth interpreted as I have already suggested in terms of protein synthesis must be in essence a surface phenomenon. The fact that the fabric theory of protein structure implies that all proteins are surface distributions of atoms when in the native state is not without interest in this connection.

MULLER: I should like to ask Dr. Wrinch whether she would still adhere to her suggestion, made in the abstract printed in the PROCEEDINGS OF THE SEVENTH INTERNATIONAL GENETICS CONGRESS, EDINBURGH (subsequently published in full in JOURNAL OF GENETICS), that possibly the reduplication of the gene takes place in a molecule that has temporarily opened out.

WRINCH: I think this view might be worth considering. However, I have since been visualizing the possibility that protein synthesis occurs at the surface of native proteins, possibly in the type of structure I have now proposed for cytoplasm in the crevices or nests associated with the branch point protein units, a suggestion which fits with certain results in enzyme chemistry (see discussion of Fruton's paper, page 216).

BANTA: I do not think we should expect Dr. Wrinch to explain so much with her hypothesis at this stage of knowledge. She would be justified in saying, "I can't explain everything."

KIMBALL: If the cell membrane is broken, protoplasm flows out macroscopically. How does this occur in your system?

WRINCH: The structure is flexible and contains so much water that flow would be expected, if the membrane is broken.

KIMBALL: So you think this molecular model makes up a unit of large size?

WRINCH: There seems at present no way of knowing whether the cytoplasm forms one system or many independent systems: it is therefore impossible to say anything about size. However a system which contains so high a complement of water must necessarily have fibrils of considerable length and permit streaming.

CHILD: What type of specific experiment would you suggest to indicate a more definite type of structure?

WRINCH: I suggest two lines of experimentation: 1) The film technique, evidently a very important line of approach since practically all important cell process take place at surfaces. 2) The X-ray approach, insofar as it can be used to give information of linear and areal arrays of molecular units.

MULLER: Would you insist that the fibers must be in a continuous net or might they also be discontinuous, as Seifritz, for example, thought?

WRINCH: I think it would be premature to make suggestions with regard to individual cytoplasmic structures in more precise terms. I have been con-

cerned rather to suggest a general type of structure which has the appropriate ratio for proteins and water. A first requirement for pressing this further would be a complete chemical composition for cytoplasms of different types. This so far I have not been able to find in the literature.

FANKUCHEN: Low angle X-ray scattering might tell whether such fibers exist. Is there any study of streaming of cytoplasm by physical techniques?

WRINCH: I understand that certain preliminary studies of this kind have already been made by Seifritz and others.

SCHULTZ: The situation could be more precisely defined by considering what we already know about different types of cytoplasm. For example, the cytoplasmic structure of muscle fibers and sea urchin eggs differ considerably. Both contain however fibrous proteins, about which we must know more in relation to the other components before their own role in cytoplasmic structure is clear. It is worth noting here that Frey-Wissling has made an attempt in some detail to derive the different structures of the cell from their molecular components.

WRINCH: I agree with Dr. Schultz that it would be of great interest to consider what is already known about different types of cytoplasm in relation to the structures here proposed. I would be particularly interested if Dr. Schultz would indicate how to work the nucleotides into the type of system I have proposed.

STERN: Do you mean by cytoplasm, protoplasm exclusive of the nucleus or exclusive of the chromosomes?

WRINCH: I have devoted my attention more particularly to the non-nuclear portion as I wished my paper to supplement the many discussions relating to the nucleus already presented in the Symposium.

CARLSON: Is there anything in the molecular model which would account for the cytoplasmic streaming in plant cells or amoeboid movement?

WRINCH: The high degree of flexibility in the model suggested and the length of the molecular

fibrils should account for cytoplasmic streaming.

CHILD: Chambers has used the microdissection technique to study such properties of the cytoplasm, and he finds that the microneedle does not pull the cytoplasm as it should on the basis of your rigid molecules.

WRINCH: Could this not be explained in terms of the models?

COHEN: How does your rigid structure fit in with the picture of rapid atomic transfer of Schoenheimer and Rittenberg?

WRINCH: I think the experiments you mention definitely imply that the native protein molecule has a highly organized structure in which the skeletal atoms of the residues are multiply connected for in this way one can explain the rapid replacement and interchange of individual atoms or building stones in a protein. These experiments in fact throw into relief the immense difference between the classical polypeptide chain theory and the fabric theory. On the first theory the nitrogen exchange would be like cutting a string into two pieces which might or might not join up again. In the second case we have the much more plausible picture of a small portion of a fabric being abstracted and replaced much as a cigarette burn on a material might be patched without danger of the whole structure falling to pieces.

FANO: I wonder whether forces analogous to those considered by Mark, which tend to keep constant the length of a flexible chain, would not cause the structure suggested by Dr. Wrinch to have a certain rigidity.

FANKUCHEN: Dr. Mark was considering changes in spatial interarrangement of atomic chains. Dr. Wrinch is considering another situation, namely, spatial interarrangement of rigid units connected by short flexible chains. There seems little relation between the two cases.

WRINCH: It seems to me that Dr. Fankuchen has put the situation very clearly. The rigidity is associated with the individual protein units and the flexibility with their interlinks.

PHYSICAL CHANGES IN THYMONUCLEIC ACID INDUCED BY PROTEINS, SALTS, TISSUE EXTRACTS, AND ULTRAVIOLET IRRADIATION

JESSE P. GREENSTEIN AND WENDELL V. JENRETTE¹

The extensive and ingenious studies of Caspersson (Caspersson, 1936; Caspersson, Hammarsten and Hammarsten, 1935) have revealed the nucleic acid-protein distribution in the chromosomes during certain phases of cell development. The intramolecular structure of both ribose- and desoxyribose nucleic acids has received attention from a host of workers, notably Levene (Levene and Bass, 1931), Thannhauser (Thannhauser and Angermann, 1929), Feulgen (1890), and Brederick (Brederick and Muller, 1939). In contrast to the considerable literature from organic chemical and biological sources, the reported studies on the physical chemistry of the nucleic acids have been very meager. Hammarsten (1924) described certain of the physical properties of thymonucleic acid, and later in a brief note with Signer, Caspersson and Hammarsten (1938) made the observation that sodium thymonucleate was highly polymerized and showed intense double refraction of flow in aqueous solution.

In the endeavor to characterize this important substance more fully, the present series of investigations on certain of the specific properties of sodium thymonucleate were undertaken. The streaming birefringence of sodium thymonucleate was so intense as to be readily revealed by the gentle stirring of an aqueous solution between crossed Polaroid disks. This property made us suspect that these solutions might also show the phenomenon of structural viscosity, and this was indeed the case. The invariable correlation of the properties of streaming birefringence and structural viscosity was confirmed by means of parallel investigations on the effect of salts, proteins, tissue extracts, and ultraviolet irradiation (Greenstein and Jenrette, 1940 and in press; Hollaender, Jenrette and Greenstein, unpublished).

THE PREPARATION OF SODIUM THYMONUCLEATE

The Feulgen-Levene procedure for the isolation of nucleic acid from tissues consisted in heating the latter with strong alkali followed by acidification, filtration of the denatured proteins, and precipitation of the nucleic acid in the filtrate with alcohol. The several preparations from calf thymus which we obtained by this method were invariably contaminated with protein and were slightly colored.²

¹ This paper, with the exception of the section on ultraviolet irradiation, has been abstracted from publications by the authors in the Journal of the National Cancer Institute 1 (1940-1941).

² Hyflow supercel was used to clarify the hot tissue extracts instead of the colloidal iron recommended by Levene and Bass (1931).

Moreover, they were soluble in water only on heating, and on cooling the solution frequently gelled. This type of preparation, while apparently quite suitable for studies of intramolecular structure (Levene and Bass, 1931), was clearly inappropriate for physico-chemical investigations. Consequently the newer preparation of calf thymus nucleate by the Hammarsten-Bang procedure was employed (Hammarsten, 1924). This procedure leads to a pure white, practically protein-free preparation, which separates from solution on treatment with alcohol as long fibers of a peculiar asbestos-like appearance. This material was easily soluble in cold water, yielding clear viscous solutions which showed intense double refraction of flow. Two preparations of sodium thymonucleate according to Hammarsten

TABLE 1. THE VISCOSITY AND STREAMING BIREFRINGENCE OF AQUEOUS SOLUTIONS OF SODIUM THYMONUCLEATE AS A FUNCTION OF CONCENTRATION (Temperature 30° C.)

Preparation No.	Concentration	Specific viscosity*	η_{sp}	Streaming birefringence
	Percent			
1 (HAMMARSTEN)	after 1.0	12.55	2024	+++
	.5	6.53	2106	++
	.25	3.54	2284	+
	.10	1.52	2451	—
2 (HAMMARSTEN)	after .5	7.46	2407	++++
	.25	4.43	2858	+++
	.10	2.08	3355	++

* Extrapolated from the viscosity- β curves for very high values of β .

were made (preparation No. 1, N 15.0, and P 8.5; preparation No. 2, N 15.1, P 8.8). Although both preparations were made in as nearly identical a fashion as was possible, the values for the viscosity and double refraction of flow were appreciably different for the two preparations (table 1). It is possible that very slight variations in the preparative procedures may be reflected in considerable differences in the physical constants of different products, even though the analytical data are the same for all of them. The difficulty in obtaining different preparations of substances of very high molecular weight which yield exactly reproducible physical values is recognizably great (Edsall and Mehl, 1940; Frampton, 1939).

A comparison between solutions of the Feulgen-Levene and the Hammarsten-Bang preparations of calf thymonucleate revealed that the birefringence and viscosity of the latter preparations were ap-

proximately three to five times greater than the former. These results are consistent with the experiments of Hammarsten (1924) in which he described the deleterious effects of alkali on the physical properties of sodium thymonucleate (cf. Schmidt and Levene, 1938). On the other hand, the relative effect of various salts on the viscosity and streaming birefringence of both types of preparation, described later, were essentially similar.

It may be emphasized that we are dealing here with the completely neutralized tetrasodium salt of nucleic acid (Hammarsten, 1924). The aqueous solutions of this salt are very slightly on the acid side of neutrality. Solutions of the free acids are too unstable to be kept for long.

THE MEASUREMENTS OF DOUBLE REFRACTION OF FLOW AND OF VISCOSITY

Double Refraction of Flow

The streaming birefringence of solutions of sodium thymonucleate was observed between crossed Polaroid disks in the apparatus described by Edsall and Mehl (1940). When the solutions were at rest the field was uniformly dark; stirring of solutions of moderate concentration caused the field to brighten; and when a rotary motion was applied, a dark cross of isocline appeared against the lighted field. When the stirring suddenly ceased, the cross of isocline very quickly vanished and the field almost immediately darkened. This is in marked contrast with the behavior of a comparable solution of rabbit myosin, because in the latter the cross of isocline persists for an appreciable length of time after stirring has ceased (Edsall and Mehl, 1940, and personal observations). In general, the addition of salts to a solution of sodium thymonucleate resulted in a diminution in the intensity of the streaming birefringence. With high concentrations of certain salts this property completely disappeared. The concentrations of salt just necessary to destroy the double refraction depend upon the nature of the salt and upon the concentration and type of preparation of the sodium thymonucleate.^a Obviously the greater the concentration of the latter and the greater its intensity of birefringence the higher will be the concentration of salt necessary to destroy the birefringence. The first investigation of these phenomena was conducted on a 0.5 percent solution of sodium thymonucleate prepared according to Levene and Bass (1931). The following results describe the concentration in millimols added per gram of solution necessary to destroy instantly the double refraction of the nucleate solution; guanidine HI 1.1, guanidine HSCN 1.2, guanidine HBr 1.4, KI 2.4, NaSCN 3.0, guanidine HCl 3.2, KBr 4.2, urea 12.4. The influence of the nature of the salt

^a We mean by the phrase, "to destroy the double refraction," only that the double refraction has been reduced to such an extent as to be no longer observable by means of the apparatus used.

is clearly evident. The remarkable effect of the guanidinium ion is especially noteworthy and will be discussed in more detail later. It may be pointed out that the concentrations of the salts mentioned above are much lower than those necessary to destroy the double refraction in sodium nucleate solutions prepared according to Hammarsten. (See table 2.)

TABLE 2. THE VISCOSITY AND STREAMING BIREFRINGENCE OF SOLUTIONS OF SODIUM THYMONUCLEATE IN VARIOUS SALTS

(The nucleic acid solution was 0.5 percent.
Temperature 30° C.)

Preparation No.	Salt	Concentration of salt	Specific viscosity*	η_{sp}	Streaming birefringence
		<i>mM added per cc. of solution</i>			
			6.53	2106	++
	NaCl	1.0	4.52	1458	+
	NaBr	1.0	4.34	1400	+
	NaI	1.0	4.17	1345	+
	Guanidine HCl	1.0	4.19	1351	+
	Urea	1.0	5.23	1687	+
	Glycine	1.0	4.92	1587	+
	NaCl	4.0	3.14	1013	+
	NaBr	4.0	2.86	923	+
1	NaI	4.0	2.48	800	+
	Guanidine HCl	4.0	2.63	848	+
	Urea	4.0	3.81	1229	+
	NaBr	6.0	2.30	742	+
	NaI	6.0	1.71	551	-
	Guanidine HCl	6.0	2.27	732	+
	Urea	6.0	3.66	1180	+
	NaBr	8.0	2.01	648	+
	NaI	8.0	.90	290	-
	Guanidine HCl	8.0	1.72	555	-
	Urea	8.0	3.34	1077	+
			7.49	2416	++++
2	Guanidine HCl	4.0	4.53	1461	++
	Urea	4.0	6.67	2151	+++
	Guanidine HCl	8.0	3.12	1007	+
	Urea	8.0	5.64	1819	++

* Extrapolated from the viscosity- β curves for very high values of β .

REVERSIBILITY OF THE SALT EFFECT

Particularly striking is the ready reversibility of the salt effect on the streaming birefringence. An apparently completely isotropic solution of sodium thymonucleate was prepared by adding any one of the above-mentioned effective salts; when such a solution was either dialyzed until salt-free, or else treated with excess alcohol to precipitate the nucleate and the latter further purified by frequent precipitation, the resultant aqueous solution in either case showed nearly the same intensity of double refraction as did the original solution of nucleate without salt. This reversibility has been repeatedly demonstrated. It stands in marked contrast with the behavior of myosin, for apparently it has not been possible as yet to restore the double

refraction of solutions of this protein when once lost (Edsall and Mehl, 1940).

VISCOSITY

The relative viscosities of solutions of sodium thymonucleate were measured at $30^\circ\text{C} \pm 0.01$ in a capillary viscometer of the Bingham and Jackson type (1917). All measurements were made under known external pressures which permitted various rates of flow of the solutions in the viscometer. The pressures were varied by means of compressed air and a system of water traps and were measured by either a water or a mercury manometer depending upon the magnitude of the pressure employed (Bingham and Green, 1919). The radius of the capillary, 0.047 cm., was found by measuring the time of flow of water under a known pressure and subsequently applying Poiseuille's law. In this connection, the value of Bingham and Jackson (1917) for the absolute viscosity of water at 30° was adopted. The volume of fluid used in the viscometer was always 5.0 cc. The salts studied were NaCl, NaBr, NaI, guanidine HCl, urea, and glycine. Two different preparations of sodium thymonucleate, made according to Hammarsten, were used, preparations Nos. 1 and 2.

The salt solutions were prepared by adding the dry salt in various quantities to a known volume of an aqueous solution of sodium thymonucleate. Similar solutions of the salts in distilled water were made for comparison. The densities of the salt solutions were not determined since relative measurements were made, and the contribution of the sodium nucleate to the density was quite negligible in comparison with that of the salt. *Fresh solutions were invariably prepared before every run.*

The pressure-time products for the salts in water were quite constant over the range of pressures employed (Appleby, 1910). The values of this product for the same concentration of salts were different, however, for different salts. At comparable concentrations the order of magnitude was as follows: glycine > NaCl > NaBr > NaI > guanidine HCl > urea. The data for the sodium salts are consistent with those of Getman for the corresponding ammonium halides (Getman, 1908).

The pressure-time product for solutions of sodium thymonucleate in water, however, were not independent of the applied pressure except for either very dilute solutions or for very high pressures. In short, solutions of thymonucleate in water do not follow Poiseuille's law. They exhibit structural viscosity to a marked degree. The progressive addition of salts to such solutions results in a distinct and progressive decrease in the viscosity.⁴ Certain salts

⁴Hammarsten (1924) showed clearly that salts will lower the viscosity of sodium thymonucleate solutions. His data were apparently obtained at a single pressure and were not sufficiently extensive to reveal the effects brought about by different salts.

in sufficient concentration are capable of destroying entirely the structural character of the viscosity. When this phenomenon occurred and the resulting solution of sodium nucleate and salt apparently followed Poiseuille's law, it was found that the streaming birefringence, like the structural viscosity, had completely disappeared. The two properties of streaming birefringence and structural viscosity thus appear to be closely interrelated, and may be thought of as being due to a high degree of molecular asymmetry of the sodium thymonucleate. Signer, Caspersen, and Hammarsten (1938) give an axial ratio (the ratio of the long and short axes of an ellipsoid of revolution) for this substance of 300:1, but in the absence of experimental data it is not possible to see how they arrived at this conclusion. Myosin is also a highly asymmetric molecule (Edsall, 1938), and as Edsall and Mehl (1940) show, there is a close relationship between the streaming birefringence of this substance and its structural viscosity.

Several investigators (Burgers, 1938; Eisenschitz, 1931, 1933; Guth, 1936; Kuhn, 1932, 1933; Peterlin, 1938, 1939) have indicated that there is some connection between the shape of molecules and the viscosity of their solutions, although no completely satisfactory relationship has as yet been developed. In order to formulate the present data to serve for future theoretical discussion, the experimental findings are given in terms of a quantity v which is the ratio of the specific viscosity, ηsp , to the volume fraction ϕ of the thymonucleate.

$$v = \frac{\eta sp}{\phi} = \frac{1}{\phi} \left(\frac{\eta}{\eta_0} - 1 \right)$$

(η is the viscosity of the nucleate or nucleate-salt solution, η_0 that of water or salt water as the case may be: η/η_0 is thus the relative viscosity.)

Einstein calculated that for a solute made up of incompressible, uncharged spheres v should be 2.5. Hatschek (1910) and recently Polson (1939) showed that the experimentally found values of v were nearer four. However, for nonspherical molecules this quantity is greater than 2.5, owing to the additional work necessary to rotate the molecule in the hydrodynamic field; and the more the shape of the molecule departs from that of a sphere, i.e., the greater the asymmetry, the greater must be the additional work. The measured apparent viscosity for substances of this type will consequently vary with the external pressure applied to force them through the capillary of the viscometer. At low external pressures the measured viscosity will be appreciably greater than that measured at high pressures because of the random orientation of the molecules under the former condition. At high pressures, however, the molecules will be oriented by the shearing stresses in the liquid and v will tend asymptotically to a lower limiting value v_∞ .

In order to formulate the experimentally found

values in terms of ν_{∞} , it is necessary to have further information in respect to two quantities: 1) The partial specific volume of sodium thymonucleate; and 2) the velocity gradient in the capillary. For the latter we have followed Edsall and Mehl (1940) in employing the function of Kroepelin (1929). Kroepelin describes a mean velocity gradient, $\bar{\beta}$, as a function of the volume v of liquid flowing through the capillary in time t and of the capillary radius r .⁶

$$\bar{\beta} = \frac{8v}{3\pi r^3 t}$$

For the determination of the partial specific volume of sodium thymonucleate we measured the densities of solutions of this substance at 30° in pycnometers, and obtained the specific volumes from the relation:

$$D = d + \frac{(1 - \alpha d)C}{100}$$

where D is the density of the solution and d of the solvent, α is the partial specific volume, and C is the concentration in grams per 100 gm. of solution. For solutions of the following concentrations the respective values of α were found: 2.0 percent, 0.65; 1.0 percent, 0.57; 0.5 percent, 0.65; 0.25 percent, 0.64; and 0.125 percent, 0.59. The average value of α is 0.62, and this was used in all computations of ϕ from the experimental data.

THE PROPERTIES OF SODIUM THYMONUCLEATE IN WATER

The structural viscosity of solutions of sodium thymonucleate in water is clearly shown in Figure 1. The viscosity falls off with increasing velocity gradient and appears to approach a limiting lower value. This limiting value minus one divided by the volume fraction of thymonucleate in solution was taken as equivalent to ν_{∞} as defined above. Thus:

$$\nu_{\infty} = \left(\frac{\eta s \phi}{\phi} \right)_{\beta \rightarrow \infty}$$

Values of the specific viscosity and ν_{∞} for both preparations are given in Table 1. In the last column of the table are given the relative intensities of the streaming birefringence of each solution as visually estimated. The values of ν_{∞} and the apparent intensity of the double refraction are different for the two preparations, but it is quite clear that the more viscous preparation (No. 2) possesses also a more intense birefringence. Both preparations consistently show the singular phenomenon of an apparent in-

crease in ν_{∞} with decreasing concentration of nucleate. In the complete absence of intermolecular forces, the value of ν_{∞} should be independent of the solute concentration. It is difficult, however, in the present instance to interpret the change of ν_{∞} with concentration, and in the absence of other information further discussion of this point must be postponed. The very large values of ν_{∞} for both preparations suggest a high degree of asymmetry of the solute

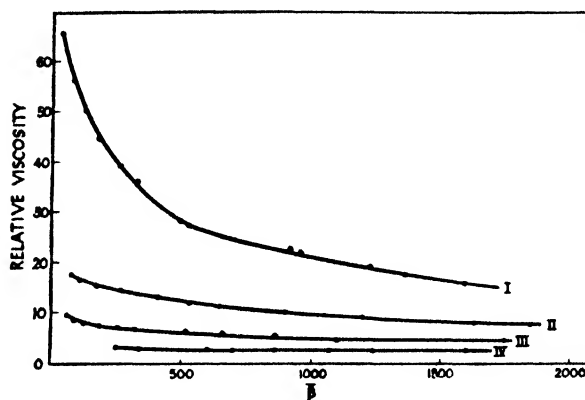


FIG. 1. Relative viscosity of sodium thymonucleate in water (preparation No. 1) as a function of the velocity gradient. I, 1.0 percent; II, 0.5 percent; III, 0.25 percent; IV, 0.10 percent.

molecules. Comparison with myosin (Edsall and Mehl, 1940) reveals that ν_{∞} for the thymonucleate is approximately 10 times as great as for the protein. It is difficult to conceive that this difference is entirely owing to differences in the magnitude of the axial ratios of the molecules of the two substances. Other factors, notably solvation, an electroviscous effect, and perhaps intense intermolecular forces may contribute to the considerable difference between the two substances. In any event, the fact that myosin is a protein and sodium thymonucleate a complex salt imposes caution on attempts to draw too close analogies, even though some striking parallelisms are present.

THE PROPERTIES OF SODIUM THYMONUCLEATE IN SALT SOLUTIONS

The profound effect which salts exert on reducing the viscosity of sodium thymonucleate is illustrated in Figures 2 and 3.

The pertinent data are shown in Table 2. The curves for the effect of 6M salts on preparation No. 1 have been omitted from the graph (fig. 2) to avoid confusion because of overlapping. There is a progressive decrease in the value of ν_{∞} for thymonucleate accompanied by an apparent decrease in the intensity of birefringence as the concentration of salt increased. In the presence of 6M NaI or 8M guanidine HCl, both the structural character of the viscosity and the double refraction disap-

⁶ Edsall and Mehl (1940) point out that the magnitude of ν is a function of the ratio of the velocity gradient, β , to ϕ , the rotary diffusion constant (Boeder, 1932; Williams and Cady, 1934). The latter quantity has not yet been determined for sodium thymonucleate, but the results in the present paper suggest that it must be relatively small.

peared (Table 2). The value of ν_{∞} under these conditions decreased to about one-fourth of its value in water; in 8M NaI it dropped to about

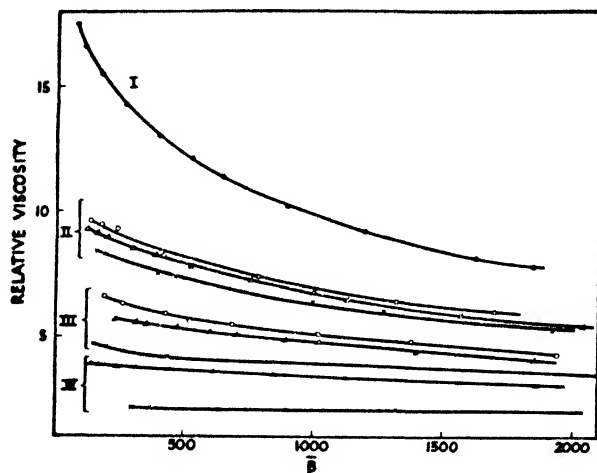


FIG. 2. Relative viscosity of 0.5 percent sodium thymonucleate (preparation No. 1) in salt solutions as a function of the velocity gradient. I, in water alone; II, in 1M salt; III, in 4M salt; IV, in 8M salt.

○ NaCl, △ NaBr, × NaI

one-seventh. The data for both preparations Nos. 1 and 2 showed the general properties of the salts, but the extent of the effect was smaller for preparation No. 2 which possessed an originally greater viscosity and a more intense birefringence. At each concentration, from 1M to 8M, the relative effect of the various salts in diminishing the specific properties of the thymonucleate solution remained the same. At comparable concentrations the order of effectiveness in this respect was as follows: NaI > guanidine HCl > NaBr > NaCl > glycine > urea. These results are consistent with those obtained with sodium thymonucleate prepared according to Levene and described above.

Examination of the curves in Figure 2 and the data in Table 2 reveals that the quantitative differences between the various salts invariably tend to increase as the concentration of the salts increase. Whereas, for example, at 1M there is almost no difference between the effect of NaI and guanidine HCl, at 4M the difference is appreciable and at 8M the effect of NaI is nearly double that of guanidine HCl. At very low concentrations of inorganic salts, as Hammarsten has indicated (Hammarsten, 1924), it is likely that individual differences between salts of the same valence type will disappear. The influence of specific ions is, however, most clearly revealed at high concentrations of salts. It must be borne in mind that the concentration of thymonucleate employed in these studies is also comparatively high.

A comparison of the effect of various salts on diminishing simultaneously the viscosity and double

refraction of myosin (Edsall and Mehl, 1940) with the data in this paper on sodium thymonucleate reveals some striking parallelisms. When the cation is the same, the iodide is stronger than the bromide, and the latter is more effective than the chloride. The most effective monovalent cation in diminishing the specific properties of both myosin and thymonucleate appears so far to be guanidinium. Guanidine HCl is consistently more effective than urea. Glycine affects both thymonucleate and lobster myosin.^{6,7} However, it must be pointed out that far higher concentrations of salts are necessary to produce the same effects in thymonucleate than in myosin. The lability of the latter to denaturing

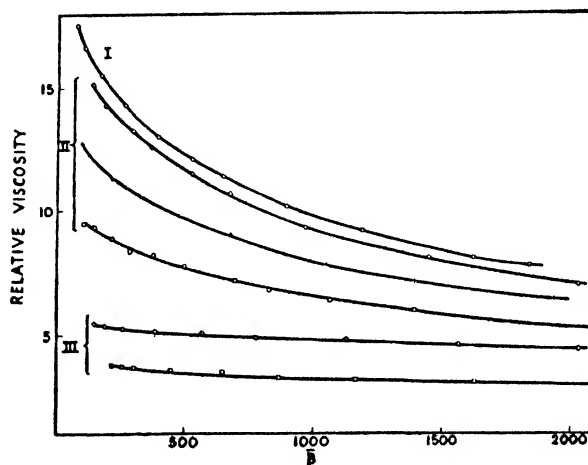


FIG. 3. Relative viscosity of 0.5 percent sodium thymonucleate (preparation No. 1) in urea, glycine, and guanidine HCl as a function of the velocity gradient. I, in water alone; II, in 1M salt; III, in 8M salt.

◇ Urea, □ Guanidine HCl, + Glycine

agents is very much greater than that of either the former or of the tobacco mosaic virus protein (Stanley and Lauffer, 1939).

* The remarkable effect of the guanidinium ion not only on thymonucleate but also on many proteins is especially noteworthy (Greenstein, 1938, 1939a and b; Greenstein and Edsall, 1940). The guanidine group is present in a wide variety of biological materials ranging from small molecules like creatine to large molecules like proteins. In all of them, because of its alkaline dissociation constant, the group exists as the guanidinium ion. The association of the nucleic acid with substances like the protamines, which are rich in guanidine groups, invites speculation. At the present time, however, it is thought best to call attention to the properties of this ion and to postpone further discussion until more experimental studies are available.

† Stanley and Lauffer (1939) have shown that the double refraction of the tobacco mosaic virus protein in 6M urea falls to about one-third the original value in 2 hours. Framp-ton 1939 has demonstrated the effect of urea in diminishing the viscosity of this protein. The behavior of the tobacco mosaic virus protein is thus similar in certain respects to those of myosin and sodium thymonucleate.

Edsall and Mehl (1940) have interpreted the effect of the various salts on myosin as being due to a dissociation of the protein molecule into smaller, less asymmetric particles. The same explanation, in part, may be tentatively applied to the analogous case of sodium thymonucleate. The latter substance has a very high molecular weight, estimated to be in the neighborhood of 1×10^6 (Hammarsten, 1939). The molecular weight of the average tetranucleotide calculated from its components amounts to about 1,400. It would therefore appear that the molecule of thymonucleate is highly associated. If the forces of association were largely of a saltlike character, i.e., between phosphate and purine amino groups, it would be expected that the addition of ionizable salts would be capable of dissolving such linkages. The comparatively strong effect of glycine and urea, however, makes such an explanation not wholly applicable. The association forces in sodium thymonucleate in any event are apparently very strong, for the latter substance is able to preserve its specific properties to some degree under such drastic conditions as boiling in alkali. Moreover, when these properties disappear in the presence of certain salts, they may be fully restored by simply removing the salt. Myosin does not possess this reversibility, and to preserve its specific properties great care must be exercised in handling the protein. It would be of great interest to investigate the possible changes of thymonucleate in particle size and shape by means of the ultracentrifuge, and plans for a study of this sort are already under way.

NUCLEIC ACID-PROTEIN MIXTURES

For the greater part, if not entirely, nucleic acid exists in the tissues in some form of combination with protein. Whether this combination is of a saltlike character, or whether it is due to an ester linkage is as yet unknown and may well be different for different proteins. The method of separation of nucleic acid from the protein offers some clue to the type of combination. For example, in the Hammarsten-Bang procedure, the neutral thymus nucleoprotein solution is saturated with NaCl and the protein is precipitated as the chloride while the sodium nucleate remains in solution. This is obviously a metathetical reaction, and it is difficult to conceive of more than a possible saltlike combination between nucleic acid and protein in thymus nucleoprotein.

The behavior of mixtures of protein with thymonucleic acid and with various salts was studied. The protein employed was rat serum albumin, purified through several crystallizations with ammonium sulfate. The final solution of the protein in water was dialyzed until free of ammonium sulfate and was adjusted to 2.8 percent concentration. Aliquots of this solution were treated with definite amounts of sodium thymonucleate or nucleate plus salts, as the case might be.

The first experiments were concerned with the estimation of the viscosity as a function of pressure for the protein in water alone, in the presence of urea, and in guanidine HCl. The data are collected in Table 3 and plotted in Figure 4.

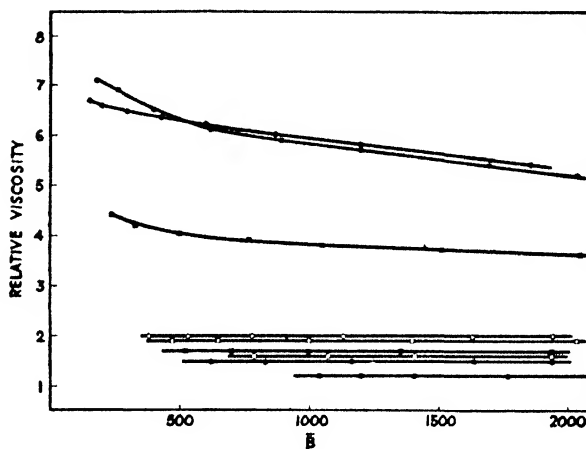


FIG. 4. Relative viscosity of 2.8 percent rat serum albumin in sodium thymonucleate (preparation No. 1) and salt mixtures as a function of the velocity gradient.

● Protein in water, ◆ in 8M urea, ■ in 8M guanidine HCl.

○ Protein plus 0.1 percent sodium thymonucleate in water, ◇ in 8M urea, □ in 8M guanidine HCl.

Top three curves refer downward to mixtures of protein plus 0.5 percent sodium thymonucleate in water, in 8M urea, and in 8M guanidine HCl.

It is evident that the viscosity of the protein in water is independent of the external pressure applied, i.e., the solution follows the law of Poiseuille. Frampton (1939) has reported the same phenomenon for this protein. Polson (1939) has pointed out that the viscosity of a fairly asymmetric protein in water, namely Helix hemocyanin (axial ratio 16.7), is independent of the external pressure. It is probable that a protein molecule must possess an extremely high degree of asymmetry in order to show the phenomenon of structural viscosity. Myosin and also the tobacco mosaic virus protein, as Lauffer (1938) and Frampton (1939) have shown, fulfill this condition. The serum albumin molecule, from the independent evidence of Polson (1939) and Neurath and Saum (1939), has a comparatively low degree of asymmetry, and is with propriety classed with the "globular" proteins.

The viscosity of serum albumin in the presence of salts also follows the law of Poiseuille, but the order of magnitude of the viscosity is greatly changed (table 3, and fig. 4). In 1M urea and guanidine HCl, the viscosities of the solutions increase, the increase being slightly greater in the latter salt than in the former. The effect is more clearly revealed in the presence of 8M salt, for here the viscosity of the solutions very markedly increase, and

again guanidine HCl increases the viscosity more than does urea. The relative effects on serum albumin of urea and guanidine HCl are consistent with the other criteria of denaturation employed, i.e., -SH groups appear in serum albumin dissolved in guanidine HCl but not in urea (Greenstein, 1938, 1939a and b; Greenstein and Edsall, 1940). Anson and Mirsky (1932) showed that the viscosity of the

TABLE 3. THE VISCOSITY AND STREAMING BIREFRINGENCE OF RAT SERUM ALBUMIN IN SALTS AND IN SODIUM THYMONUCLEATE

(The concentration of serum albumin was always 2.8 percent. The pH in water and in the salts was 6.0. The pH in mixtures with sodium thymonucleate was 8.0. Temperature was 30° C.)

Solvent	Salt	Concentration of salt	Specific viscosity*	Streaming birefringence
		<i>mM added per cc. solution</i>		
H ₂ O.....			0.21	—
	Guanidine HCl	1.0	.25	—
	Urea.....	8.0	.73	—
		1.0	.22	—
		8.0	.54	—
0.1 percent Na thymonucleate (preparation No. 1).....			.66	—
	Guanidine HCl	8.0	0.92	—
	Urea.....	8.0	1.09	—
0.5 percent Na thymonucleate (preparation No. 1).....			4.02	+
	Guanidine HCl	8.0	2.67	—
	Urea.....	8.0	4.13	+

* Extrapolated from the viscosity- β curves for very high values of β .

"globular" proteins is increased in urea, while Neurath and Saum (1939) showed that η for serum albumin increased from 6.50 in water to 22.6 to 6.66M urea. Recently Bull (1940) demonstrated the marked rise of the viscosity of egg albumin in urea. Neurath and Saum and Bull suggested that this increase in viscosity is owing to a change in the shape of the protein molecule in the direction of greater asymmetry. Whatever the explanation may be, these data on serum albumin and egg albumin in certain salt solutions are in marked contrast with those of sodium thymonucleate and of proteins such as myosin and the tobacco mosaic virus. The viscosity of the albumins increases, the viscosity of thymonucleate, myosin, and tobacco mosaic virus protein decreases when the same salts are added to solutions of each. The relative order of effect is also curiously preserved, thus guanidine HCl, which raises the viscosity of serum albumin more than urea, also decreases the viscosity of myosin more than urea. Without necessarily assuming any inter-

pretations, there is evidently a considerable difference in the behavior of certain "globular" proteins and certain fibrous substances such as thymonucleate toward the same salts. With this difference in mind, we investigated the behavior of mixtures of the two types of substances in the presence of these salts, with particular reference to protein and nucleic acid.

Nucleic acid was mixed in two different proportions with serum albumin, and the viscosity at different pressures determined in water alone, in the presence of 8M urea, and in the presence of 8M guanidine HCl. When the sodium thymonucleate was added to the isoelectric protein, a heavy precipitate of the mixture appeared. Addition of sodium hydroxide solution to pH 8.0 resulted in a clear solution, and measurements were necessarily performed under this condition. The thymonucleate was added in such amount that in one mixture it was about 3.6 percent of the protein, and in the other about 18 percent. If each component of the mixture reacts individually with the added salt, it would be expected that the effect of the salt in increasing the viscosity of the protein would be counterbalanced by the effect of decreasing the viscosity of the thymonucleate. The resultant viscosity would be a function of the algebraic sum of the two effects. The data are given in Table 3, and the curves relating the viscosity-velocity gradient function are given in Figure 4.

The first noticeable effect is that the value for the viscosity of the nucleic acid-protein mixture is between that of the thymonucleate and the protein considered separately (tables 1 and 3). The effect of the protein at 2.8 percent concentration (approximately 4×10^{-4} molar) in diminishing the specific viscosity of 0.5 percent thymonucleate from 6.53 to 4.02 is very much greater than the decrease to 4.92 brought about by one M glycine (tables 2 and 3). These and subsequent effects are much too large to be ascribed to the comparatively small effects caused by changes in pH. When urea and guanidine HCl are added to a mixture of 0.1 percent thymonucleate and protein there is a distinct rise in the viscosity of the mixture, but in contrast with the case of the protein alone, the viscosity in urea is greater than in guanidine HCl. The reason is due to the fact that whereas the salts simultaneously increase the viscosity of the protein and decrease the viscosity of the thymonucleate, the effect on the protein is greater than that on the thymonucleate. The relative increase in the viscosity of the mixture is greater in urea because the contribution to the total viscosity owing to the thymonucleate is lowered more by guanidine HCl than by urea (table 2). When 0.5 percent thymonucleate is mixed with the protein, the relative decrease of the viscosity of the thymonucleate is smaller than when the ratio of protein to thymonucleate is higher. The contribution to the total viscosity of the mixture by the higher concentration of thymonucleate predomi-

nates; and thus when guanidine HCl is added, the specific viscosity of the mixture drops from 4.02 to 2.67. That the contribution of the protein is still appreciable is indicated by the effect of 8M urea, for in the presence of this salt the viscosity of the mixture increased slightly (table 3). In the latter case, even in the presence of a large amount of thymonucleate, 8M urea lowered the viscosity of the latter to a less extent than it increased the viscosity of the protein.⁸

Finally, the intensity of the streaming birefringence of 0.5 per cent thymonucleate was diminished but still apparent in mixtures of the latter with protein in water and in 8M urea. In 8M guanidine HCl is disappeared.

It must be emphasized that the foregoing results were obtained on definite mixtures of protein and nucleic acid. Each component in these mixtures acts as a separate entity.

THE EFFECT OF ULTRAVIOLET IRRADIATION

(In collaboration with Dr. Alexander Hollaender)

A 0.5 percent aqueous solution of sodium thymonucleate (part of preparation No. 2 described above) was prepared and divided into two equal parts. One part was placed in a quartz tube and irradiated by a lamp delivering radiation, 80 percent of which is at 2537 Å. The other part was placed in a glass tube and allowed to remain under the same conditions as the contents of the irradiated tube. At selected time intervals an aliquot was removed from each tube and the relative viscosity determined at varying external pressures. Simultaneously, the extent of the streaming birefringence was visually estimated. The data are revealed in Figure 5.

The abscissa, β , in the figure is a function of the velocity gradient, and has been defined above. The dependence of the viscosity values of the non-irradiated thymonucleate solution on the velocity gradient at low values of the latter is revealed in Figure 5. There is a small but unmistakable decrease in this dependence with standing, due most likely to a slight spontaneous breakdown of the thymonucleate structure. No such decrease of the viscosity values with time is apparent at the higher velocity gradients, indicating the very slight extent of the spontaneous changes involved. No significant alteration in the intensity of the streaming birefringence could be visually estimated. On the other hand, the dependence of viscosity on the external pressure is greatly decreased in the case of the irradiated solutions (fig. 5). This dependence furthermore decreases with the time of irradiation, until after about 83 hours the viscosity is practically independent of the velocity gradient. Simultaneously with the decrease in the structural charac-

ter of the viscosity function for the irradiated solutions, the streaming birefringence also diminishes in intensity. After the 83-hour period of irradiation, both properties disappeared. The pH of the irradiated solutions, 6.90, was the same as that of the controls, and free phosphate or ammonia was completely absent. The absorption curve of the irradiated thymonucleate was identical with that of the original material, thus indicating that no extensive destruction of the ring structures had occurred.

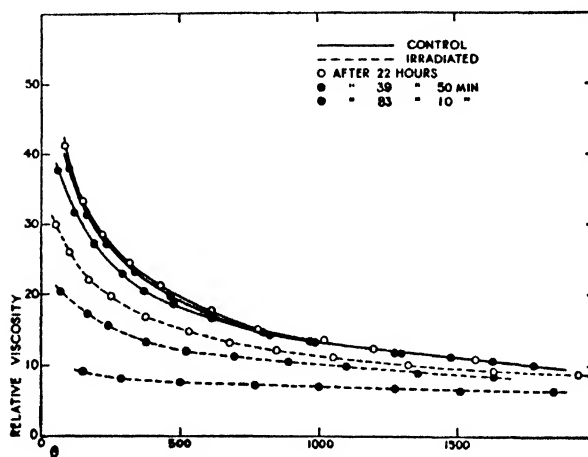


FIG. 5. The viscosity of irradiated solutions of thymonucleate.

The loss of these specific physical properties of thymonucleate on ultraviolet irradiation may be interpreted as a depolymerization of the highly asymmetric particles into smaller, less asymmetric fragments. Such an explanation has already been advanced to account for the loss of these properties in thymonucleate by proteins and salts (Greenstein and Jenrette, 1940) and in myosin by denaturing agents (Edsall and Mehl, 1940). Svedberg and Brohult (1939) have found that the molecular weight of Helix hemocyanin is reduced by ultraviolet irradiation. The extent of the depolymerization of the irradiated thymonucleate has not yet been exactly determined; ultracentrifugal measurements to determine this are now in progress.

THE TIME COURSE OF THE REACTION OF THYMONUCLEATE WITH PROTEINS, SALTS, AND TISSUE EXTRACTS

Historical and Critical Survey

The older method of isolating thymonucleic acid from tissues has been to boil the minced tissues with strong alkali, followed by acidification, removal of the denatured proteins by filtration, and precipitation of the nucleic acid from the filtrate by addition of alcohol. This procedure leads to a granular product which dissolves in hot water. On cooling, the solution gels. Feulgen (1935) found that a longer period of boiling the minced tissues with strong

⁸ The viscosity in solution of .5 percent thymonucleate in the presence of the protein is still structural (fig. 4).

alkali resulted in obtaining a product which dissolved in hot water, but the solution failed to gel on cooling. He called the former preparation of thymonucleic acid the a-form, the latter the b-form. It seemed likely that the latter form was a degradation product of the former, and Feulgen himself admitted in a fairly recent publication the "schlechten Rufe" in which the b-form was regarded by many workers in the field. However, the a-form, which causes gelation of its aqueous solutions, was not above suspicion for it was inconceivable that a product isolated under such drastic conditions would be representative of conditions in the intact living tissue. Indeed, Hammarsten in 1924 showed that the a-form of Feulgen was an artificial product of the action of strong alkali on "native" thymonucleic acid. The preparation of "native" nucleic acid in the highly polymerized form in which it presumably occurs in tissues was accomplished by Hammarsten by a process employing salt precipitation in the cold. The nucleic acid isolated by this method appears in the form of long white fibers which dissolve readily in cold water without gelling.

In spite of the purity and obvious advantages of the Hammarsten preparation of thymonucleic acid very few workers outside of Sweden concerned themselves with it. It was ignored by Feulgen, and only in 1939 was a study published comparing the various preparations of thymonucleic acid. Schmidt, Pickels, and Levene (1939) stated that the degree of polymerization of the preparations of thymonucleic acid by various workers decreased in the following order: Hammarsten, Neumann, Levene, Feulgen. In 1935, Feulgen, who was still concerned with the a- and b-forms of thymonucleic acid, announced the presence of an enzyme in pancreatin powder which transformed the a- to the b-form. According to Feulgen no phosphorus was thereby split from the nucleic acid. Feulgen named this enzyme "nucleogelase." Its action was indicated by the transformation of the gel (a-form) to the watery consistence of the b-form. Schmidt, Pickels, and Levene stated that the Hammarsten preparation of thymonucleic acid would not yield phosphorus by the action of intestinal phosphatase until it had been previously "depolymerized" by Feulgen's method with pancreatin.

The evidence offered by Feulgen and by Schmidt, Pickels, and Levene for the presence of a depolymerizing factor in pancreatin was indirect. A transformation of gel to sol, or the action of phosphatase is not evidence for depolymerization. There are salts and inert proteins in the commercial preparations of pancreatin. Greenstein and Jenrette (1940) showed that pure proteins and a wide variety of salts have the property of depolymerizing nucleic acid. A small amount of a strongly active salt such as guanidine hydroiodide immediately converted a gel of thymonucleic acid (Levene preparation) to the sol form. Greenstein and Jenrette followed the extent of the depolymerization brought about by

these agents by the diminution of the structural viscosity and the streaming birefringence of the thymonucleic acid. Again, when Schmidt, Pickels, and Levene (1939) incubated the thymonucleic acid with pancreatin for two days at 37°, no mention was made of the spontaneous depolymerization which the nucleic acid undergoes at an elevated temperature during a long period of time.

In addition to the omission of direct physical evidence for the depolymerization brought about by the "nucleogelase" enzyme of Feulgen, this investigator also failed to establish the necessary criteria for the presence of an enzyme. These consist, among other things, in the time course of the enzymic reaction, and in the use of inhibiting agents, more or less specific for enzymes, which affect the velocity of such reaction.

In view of the insufficient evidence for the presence of a depolymerizing enzyme described above, it was considered desirable to reinvestigate this problem, so important for a better understanding of the mechanisms involved in nuclear processes. A study was made of the time course of the reaction of thymonucleic acid with extracts of normal and cancerous tissues. The study of lactating breast led to an extension of the investigation to the milk and the sera of several species. In order to assist in the interpretation of the results obtained, the observations by the authors (1940 and in press) on the depolymerization of nucleic acid by proteins, amino acids, and salts were extended to a consideration of the effect of these agents over a definite period of time.

Total nitrogen determinations were made on aliquots of each of the tissue extracts, protein solutions, and samples of sera and milk studied. Before the effects of these fluids were investigated on nucleic acid, they were adjusted with distilled water to definite and equal concentrations of total nitrogen in mg. per cc. By comparing the effects of these fluids at equal concentration of total nitrogen a certain uniformity in the experimental conditions is achieved. The total nitrogen figure for each solution indiscriminately includes that due to proteins, urea, amino acids, ammonium salts, etc. All of these components affect nucleic acid (Greenstein and Jenrette, 1940), and all vary in their absolute amounts and relative proportions in the fluids studied. To place each of these fluids on an equivalent total nitrogen basis is obviously a somewhat arbitrary procedure; nevertheless it is difficult to conceive at the present time of a better method of placing the comparison of the fluids on a uniform basis. At least two and sometimes three different concentrations of total nitrogen were chosen. Several samples of each tissue were studied. The reproducibility of the results obtained were satisfactory throughout except in the single case of the lower concentrations of liver and hepatic tumor extracts. No reason can be advanced at the present time for this discrepancy. Included is a comparison of the effect of the whole spontaneous

mammary tumor in C₃H mice and the mammary tumor in this strain from which the necrotic areas were carefully dissected.

The substrate upon which all the above agents act was a preparation of sodium thymonucleate prepared according to Hammarsten. A description of this particular preparation (No. 2) is given above. Its aqueous solution has a pH of 6.9. Occasionally for convenience the thymonucleate substrate may be referred to as thymonucleic acid, or nucleic acid, but it is understood that it is always the completely neutralized tetrasodium salt of thymonucleic acid to which the reference is made.

We incubated at 30°C mixtures of sodium thymonucleate with various tissue extracts, proteins, amino acids, and salt solutions, and samples of milk and of sera in the viscometer itself and measured the viscosity of the mixture at selected intervals. An external air pressure of 16.0-cm. H₂O was employed to force the solution through the capillary.⁹ This pressure was selected because it occurs within the range where the viscosity of the thymonucleate is most sensitive to changes in its molecular shape. Simultaneously, a portion of the mixture was examined from time to time for its streaming birefringence in the Edsall-Mehl apparatus.

The method of mixing was as follows: *A one percent solution of sodium thymonucleate was prepared fresh before every determination.* A 5 cc. portion of this solution was mixed with 5 cc. of the fluid whose effect was to be studied. Simultaneously, 5 cc. of the nucleate solution were mixed with an equal volume of distilled water. The former mixture was the test, the latter was the control. A further control was set up by diluting the fluid studied by an equal volume of distilled water. The partial volumes occupied by the nucleate and by the protein molecules were disregarded. The time of mixing was carefully noted, and selected volumes of the test and of the control mixtures were transferred to the viscometers. Readings of the relative viscosity of these mixtures were taken at various time intervals. The viscosities were calculated as usual relative to those of the extract, protein, or salt solutions as the case happened to be. The concentration of sodium thymonucleate in test and in control was 0.5 percent. At 16 cm. H₂O pressure, the time required for the control to pass through the capillary used was about nine minutes; the time similarly required for the dilute extract solution was about 14 seconds. A considerable time-span was therefore provided, in which changes in the viscosity of the nucleate could be noted with ease and with accuracy.

Determinations of inorganic phosphorus and ammonia were performed on an aliquot of the mixture of nucleic acid and extract, and on an aliquot of the extract at the beginning and at the end of the

incubation period. It may be here stated that in no case, under the conditions used, was there any evidence of an analytically-detectable change in the phosphorus or ammonia content of the mixture after incubation. There was, likewise, no apparent change in the pH. Thus, so far as one may determine at the present time, there are no chemical changes involved under the present conditions in the mixture of nucleate and fluids used—any changes observed are apparently of a physical nature only. The failure to detect significant phosphatase activity in the mixtures used is similar to the findings of Maver and Voegtlin (1935) who showed that thymonucleic acid is very little affected by tissue phosphatase within 4 hours at pH 7 and at 37°C. These investigators showed that tissue phosphatase has a major optimum activity at pH 8.8, and a minor optimum at an acid reaction. Our working conditions include a pH range of 6.8-7.2, an incubation period of 4 hours, and an incubation temperature of 30°C. In view of the results of Maver and Voegtlin, our failure to observe any significant change in the phosphorus content is not unexpected.

The experimental results are given in the form of graphs, wherein the relative viscosities are plotted as a function of the time of incubation in the viscometers. The relative effect at equivalent nitrogen concentrations of each of the fluids studied can thus be visually compared. The order of reaction for the active preparations has not as yet been determined, and thus the comparisons drawn are of a semi-quantitative character only. However, the differences in activity, visually noted, are quite clear. In each graph the values of the two controls are given, i.e., the viscosity of a 0.5 percent solution of thymonucleate in water shown by the continuous line in each chart, and the viscosity of the appropriately diluted fluids used. The viscosity values of the latter were used exactly in the calculation of the relative viscosities of the mixtures. Since, however, the comparatively small differences between the viscosity values for the fluids are not readily revealed in the graphs, a single averaged value is used for this control and is so *represented by the broken line in each chart.* At 16 cm. H₂O pressure, no changes in the values of the viscosities of the various fluids can be observed during the incubation interval. If any changes do occur in these values, they cannot be picked up at the high velocity gradient used, and hence do not affect the values observed for the much more viscous mixtures of the fluids with thymonucleate.

THE INTERACTION WITH THYMONUCLEATE

Pure Proteins, Amino Acids, and Salts

Greenstein and Jenrette (1940) showed that proteins, glycine, and a wide variety of salts lower the structural viscosity and extent of streaming birefringence of thymonucleate. Of the salts, the most effective cation was guanidinium, the most effective

*The viscosity of a highly asymmetric molecule such as sodium thymonucleate has no meaning unless the external pressure is known.

anion was iodide. The data of this earlier study were obtained on fresh solutions within a short period after mixing. The time course of the interaction is discussed in the present study. In Figures 6 and 7 are shown the values of the viscosities of mixtures of thymonucleate with pure proteins, amino acids, and salts over 4 hours of incubation at 30°C in the viscometers.¹⁰

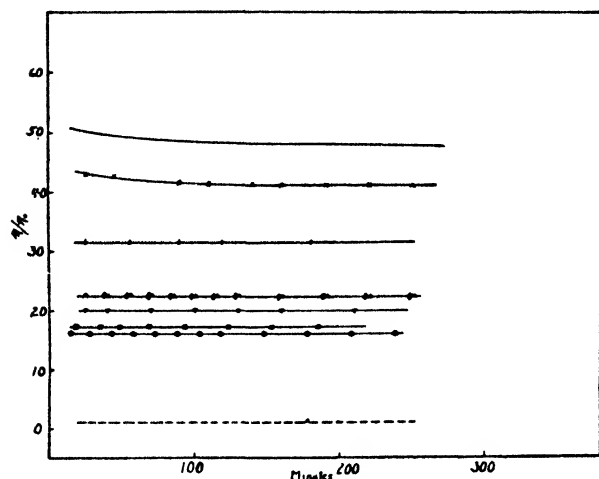


FIG. 6. The incubation of thymonucleate with inorganic and amino acid salts. Ordinate, relative viscosity; abscissa, time of incubation.

—Control, ○ 2M Guanidine HCl, □ 0.2M, ◇ 0.002M, × 0.00002M, △ 0.5M Lysine HCl, | 0.5M NaCl, — — — Control

From the curves in Figures 6 and 7, two conclusions may be drawn: *a*) the various agents used lower the viscosity of the nucleate to an extent dependent on the nature and concentration of the agent, and *b*) the rate of fall in the viscosity over the four-hour period for the nucleate in water alone and in the mixtures is almost negligible. Attention need, therefore, be only directed to the relative effect of the various agents used on lowering the viscosity values of the thymonucleate independently of the time of incubation. The remarkable effect of guanidine HCl, at a concentration as low as 10^{-5} M, reveals the properties of this salt. Sodium chloride at 0.5 M has a much weaker effect on the viscosity of thymonucleate than has guanidine HCl at 0.2 M. Arginine HCl and lysine HCl are also more effective than sodium chloride at equivalent concentrations. Arginine HCl differs from lysine HCl by the possession of a guanidine group substituted on the terminal carbon atom; the former lowers the viscosity of nucleate to a greater extent than the latter at equivalent concentrations, a result that might well

¹⁰ Guanidine HCl is best purified by repeated solution at room temperature in dry methanol followed by precipitation with equal volumes of dry ether.

be attributed to the presence of the guanidine group in the arginine molecule.

The purified proteins, crystalline egg albumin and horse serum albumin, affect the viscosity of thymonucleate (fig. 7) to a remarkable degree if the concentrations of proteins used are placed on a molar basis. Thus, a solution of horse serum albumin containing 2.46 mg. N per cc. lowers the relative viscosity of thymonucleate from about 50 to about 20. The concentration of protein in this solution is approximately 0.002 M. Yet the effect of lowering the viscosity of nucleate is very much greater than that of glycine at one M or histidine at 0.05 M. The same is true of the behavior of egg albumin. This property of proteins will be discussed below. For the present it need simply be pointed out that the effect of the proteins on lowering the viscosity of nucleate is proportional to the concentration of protein, and that at equal nitrogen concentrations egg albumin is more effective than serum albumin. Under such conditions the molar concentration of the former protein is about double that of the latter.

Histidine at 0.05 M concentration is more effective than glycine at one M, a phenomenon which justly may be ascribed to the presence of the imida-

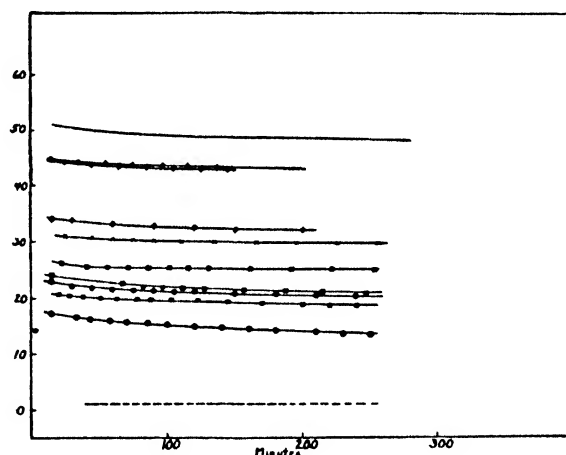


FIG. 7. The incubation of thymonucleate with proteins and amino acids. Ordinate, relative viscosity; abscissa, time of incubation.

—Control, ● Egg Albumin, 2.46 mg.N per cc., ⊗ 1.23 Mg.N per cc., ■ Serum Albumin 2.46 mg. per cc., ⊠ 1.23 mg.N per cc., □ 0.41 mg.N per cc., ◇ Glycine 1.23 mg.N per cc., ◆ IM, × Histidine 0.05M, — — — Control

zole ring in the former amino acid. However, glycine at as low a concentration as 1×10^{-4} M, nevertheless has a distinct effect in lowering the viscosity of thymonucleate.

In the case of every mixture, the streaming birefringence was distinctly positive although lower in degree than that of the control of thymonucleate in water.

EXTRACTS OF TUMORS AND NORMAL CONTROL TISSUES

The effect which tissue extracts exert on the physical properties of thymonucleate is illustrated in Figures 8 and 9. Examination of the curves in these figures reveals that two distinct steps are involved in the interaction: 1) A decrease in the viscosity brought about by the presence of the proteins and salts in the extract, a decrease which occurs almost at once after mixing; and 2) a very rapid rate of decrease within the incubation period, so rapid with certain extracts as to reduce the viscosity of the mixture to the value characteristic of the extract itself within a short space of time. The first step is simply that characteristic of proteins, amino acids, and salts as shown in Figures 6 and 7. It is suggested in Figure 8, for example, by the fact that the initial decrease (up to the first 15 minutes) is from a relative viscosity of about 50 to about 18. After this initial measured period, the rate of decrease is comparatively much slower, the viscosity dropping from about 18 to 5 in four hours. The first step is likewise suggested in Figure 9 by the curves for the lactating and hyperplastic mammary tissues, which illustrate the great initial drop in viscosity soon after mixing, followed by the rela-

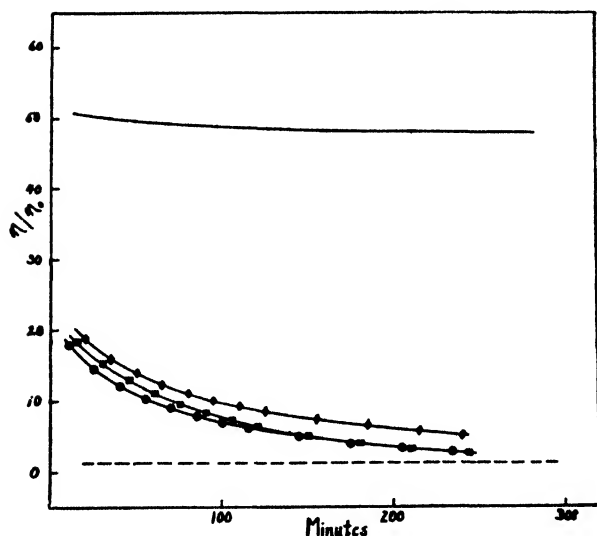


FIG. 8. The incubation of thymonucleate with rat liver extracts. Ordinate, relative viscosity; abscissa, time of incubation. Each extract contains 1.23 mg. N per cc.

— Control, ■ Normal liver, ● Regenerating liver, ◆ Hepatic tumor, — — — Control

tively slower decrease during the incubation period. Although the latter decrease in viscosity, illustrated in the curve, is lower in absolute amount than that shown by the initial drop, it is nevertheless, a phenomenon which pure proteins, amino acids, and salts do not exhibit. The effect of these latter substances on the viscosity of thymonucleate after the initial

drop is practically negligible. Furthermore, in many of the tissue extracts, the initial drop (within 15 minutes after mixing) is very much greater than that shown by an equivalent amount of pure protein. This fact is particularly illustrated by the case of the more concentrated extract of mammary tumor in Figure 9.

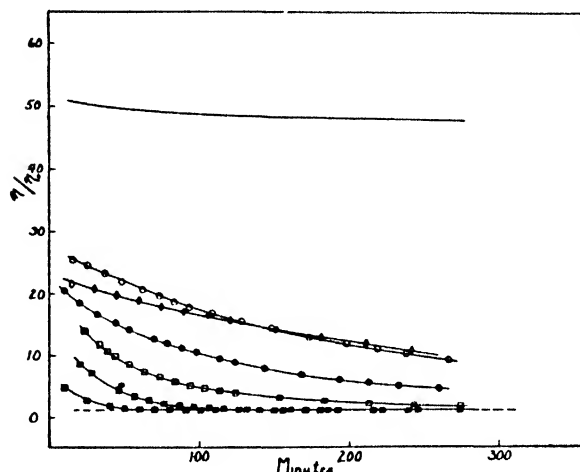


FIG. 9. The incubation of thymonucleate with mouse mammary tissue extracts. Ordinate, relative viscosity; abscissa, time of incubation.

— Control, ○ Lactating breast 0.41 mg. N per cc., ● 1.23 mg. N per cc., ◇ Hyperplastic breast 0.41 mg. N per cc., □ Mammary tumor (no necrosis) 0.41 mg. N per cc., ■ 1.23 mg. N per cc., ⊠ Mammary tumor (including necrotic areas) 1.23 mg. N per cc., — — — Control

The conclusion appears inescapable, therefore, that there must exist some factor in the tissue extracts which is not inert protein, amino acid, or salt, and which in sufficient amount appears to be capable of reducing the viscosity of thymonucleate to that almost characteristic of water. That this factor causes the disintegration of the asymmetric molecules of thymonucleate is shown by the fact that when a sufficiently low value of the viscosity of the mixture is reached the streaming birefringence disappears. There is a distinct correlation between these two properties, and later in this communication we show that at the end of the incubation periods of mixtures of thymonucleate with certain tissues, *when the viscosity of each mixture has become constant*, the structural character of the viscosity disappeared. These phenomena can be interpreted on the basis that a complete, or nearly complete, depolymerization of the thymonucleate to the tetranucleotide stage has occurred, and that the agent responsible for this is an enzyme or system of enzymes existing in the tissues studied. Thus when a solution of thymonucleate is treated with an appropriate tissue extract, two steps occur: 1) An initial drop in the viscosity and streaming birefringence due now not

only to the presence of inert protein, amino acids, and salts but also to the presence of a depolymerizing factor in the extract; and 2) a gradual but marked decrease in these properties until a minimum value is reached. The activity of the enzyme factor superimposed upon the initial drop due to the presence of proteins, etc., is responsible for the greater magnitude of the initial drop 1) in the case of tissue extracts than that shown in the case of solutions of pure proteins of equal nitrogen concentration. Further evidence in favor of the presence of an

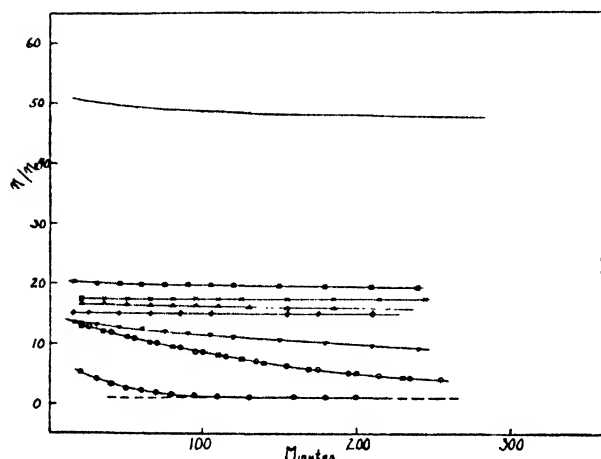


FIG. 10. The incubation of thymonucleate with milk. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 2.46 mg. N per cc.

— Control, ○ Guinea Pig, ◇ Goat, ● Rat, □ Mouse, △ Mare, × Cow, ■ Human, — — — Control. Third curve from bottom: rabbit.

enzyme factor lies in the fact that increasing the concentration of the same tissue extract in the mixture results in increasing not only the magnitude of 1), the initial drop, but also the subsequent rate of drop 2). This phenomenon is well illustrated in Figure 9. Still further evidence for the presence of the enzyme factor is adduced from experiments with serum described below.

Because of the changes in physical properties of thymonucleate brought about by the enzyme factor in the tissue extracts studied, changes interpretable only on the basis of a depolymerization, we have chosen to name this enzyme *thymonucleodepolymerase*. The exact mechanism of the action of this enzyme is as yet unknown—it is recognizable only by the physical effects which it produces. It may consist of a single factor or a number of factors. No phosphatase activity is associated with its effects, in any case under the conditions which are employed in this investigation. The relative amount of the depolymerase in any given extract of tissue can be estimated by the rate at which a standard solution of thymonucleate diminishes in viscosity and streaming birefringence.

The curves in Figure 8 and Figure 9 reveal the presence of the depolymerase in both hepatic and mammary tumors and in the normal tissues from which the tumors are derived. There are significant differences in the relative activity of the depolymerase in the various tissues. At equal concentrations of total nitrogen, the activity of the rat hepatic tumor is less than that of normal rat liver and regenerating rat liver, the activity of the latter two tissues appearing to be about equal after a short period (fig. 8).

Examination of Figure 9 reveals the very remarkable activity of the depolymerase in the extracts of spontaneous mammary tumors in C₃H mice. Particularly is this true of the whole tumor, e.g., including necrotic areas, described by the crossed-in squares in Figure 9. Fifteen minutes after an extract of this tissue is mixed with thymonucleate, the streaming birefringence of the latter disappears, and one hour after the mixing the viscosity of the mixture reaches a constant value little more than that of water. This phenomenon has been repeated over and over again with various samples of C₃H mammary tumors. When the necrotic areas are carefully removed, the extract of the remaining tissues is somewhat less than before, but still extremely active. Comparison with the activity of the control tissues, lactating and hyperplastic breast, reveals the far greater amount of enzyme in the tumors. Indeed, the activity of the mammary tumor extract which has a concentration of 0.41 mg. N per cc. is very much greater than that of an extract of lactating breast of three times this nitrogen concentration. After about two hours of incubation the activity of lactating and of hyperplastic breast appears to be nearly the same.

THE MILK OF VARIOUS SPECIES

The presence of the depolymerase in normal mouse breast led us to inquire whether it might not be present in the milk of the mouse. Milk was drawn from many animals by an aspirator developed in this institute. Investigation of mixtures with thymonucleate revealed the presence of the depolymerase in mouse milk (fig. 10). This rather surprising finding led us to investigate the milk of a number of different animals. Several samples of the milk of the human, mare, cow, and goat failed to indicate the presence of the depolymerase. The results on these samples of milk are depicted in Figure 10, where the data resemble those obtained on solutions of pure proteins. Thus, there is the initial drop in viscosity due to the proteins of the milk followed by a nearly constant value for the viscosity during the whole of the incubation period.

In contrast to the results obtained on the milk of these species, the results with the milk of the rat, mouse, rabbit, and guinea pig all revealed the presence of the depolymerase (fig. 10). The activity of mouse and guinea pig milk was practically identical.

The activity of rabbit milk was not very marked, but that of rat milk was by far the most powerful of all the samples investigated. In relative order of magnitude, the activity even of rat milk is not as great as that found in extracts of mammary tumor or normal liver. However, the fact that the presence of the depolymerase is so readily revealed in the milk of certain species indicated that its amount is not negligible. The enzyme is synthesized presumably in the mammary tissues and finds its way subsequently into the milk. Its presence in the milk of the rodents is in any case unexpected. Whether the depolymerase serves a special function in the milk, or whether the latter fluid is a vehicle whereby the young acquire a ready-made enzyme system for the first period of life, or whether the presence of the enzyme is purely adventitious, all remain for further investigation.

THE SERA OF VARIOUS SPECIES

The finding of the depolymerase in the milk of the rodents led us to investigate the sera of a number of animals of different species. The results of viscometric determinations on mixtures of sera with thymonucleate under identical conditions are depicted in Figure 11.

Inspection of Figure 11 reveals that again there is an initial drop in the viscosity of the mixtures brought about by the presence of proteins, etc., in the serum, and that this drop is followed either by a fairly constant value during the incubation period or else by a decreasing approach to a lower limiting value during this period. Sera which followed the former path were derived from the human, horse, cow, and rat, and exhibit little or no depolymerase activity. Sera which followed the latter path, presumably because of their depolymerase activity, were obtained from the dog, guinea pig, mouse, and rabbit. There appears to be something of a difference between the behavior of rat and beef serum on the one hand and that of human and horse serum on the other within the first two hours of incubation (fig. 11). It is possible that there is a very slight activity of the enzyme in rat and beef serum, which is exhibited in the early part of the incubation period, and which then levels off to a negligible value in the later stages. No trace of activity at any stage is shown in the case of human or of horse serum.

Of the sera which clearly demonstrate depolymerase activity, that of the dog is the most effective. Within an hour of mixing the dog serum with thymonucleate, the streaming birefringence of the latter disappears and the viscosity of the mixture is nearly that of the diluted serum itself.

The dog serum was separated by the usual procedures with ammonium sulfate into the three general protein fractions, albumin, euglobulin, and pseudoglobulin, and each fraction was investigated under identical conditions for the presence of depolymerase activity. Although the euglobulin frac-

tion of dog serum was the only one which showed significant depolymerase activity, it was in no way as active as the whole serum. It would seem as if a considerable proportion of the activity of the serum was lost during the fractionation procedure. Recombination of the various fractions failed to restore the activity characteristic of the whole serum. However, quite apart from the absolute magnitude of the activity, it is sufficiently interesting to note that qualitatively the activity is characteristic of one of the protein fractions of the serum.

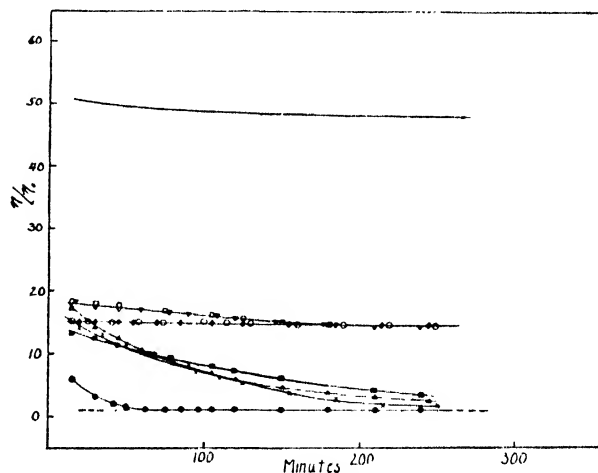


FIG. 11. The incubation of thymonucleate with serum. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 2.46 mg. N per cc.

—Control, ● Dog, ◇ Human, ○ Horse, □ Beef, △ Guinea Pig, ■ Mouse, × Rabbit, — — — Control. Second curve from top: rat.

The various sera which were studied were obtained separately in many cases from males and females. No significant sex differences in the results were obtained either in the case of human serum which does not contain the enzyme or in the case of rabbit serum which contains the enzyme.

Attempts were made to see whether treatment of human and rabbit sera with oxidizing and with reducing agents would have any effect on the catalytic depolymerization of thymonucleate. These sera were treated respectively with neutralized cysteine and with an excess of porphyrindin, and allowed to stand for one hour before mixing with thymonucleate. The sera treated with cysteine stood in an evacuated Thunberg tube. The color of the added porphyrindin faded to a light orange during the period of standing. The results, however, on human and rabbit sera treated in this fashion were identical with those obtained on untreated sera.

In order to determine whether a dialyzable factor was present in the inactive human serum and in the active rabbit serum, samples of these sera were dialyzed against distilled water in the cold room at 5°C for two weeks. The results, however, on the

dialyzed preparations were identical with those obtained on undialyzed sera.

In order to determine whether there was any inhibiting factor to the depolymerase in an inactive serum such as that of the horse, the effect of a mixture of horse serum with active rabbit serum was investigated. A mixture of the two sera was prepared in which each was present to the concen-

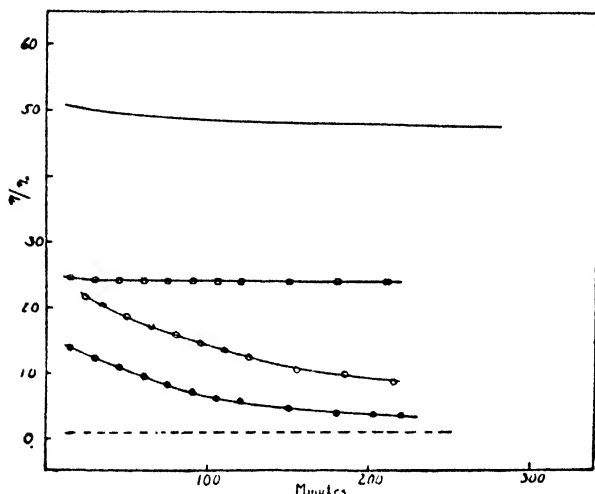


FIG. 12. The incubation of thymonucleate with a mixture of horse and rabbit serum. Ordinate, relative viscosity; abscissa, time of incubation.

— Control, ○ Rabbit 1.23 mg. N per cc., □ Horse 1.23 mg. N per cc., ● Mixture containing 1.23 mg. N per cc. of each component.

tration of 1.23 mg. N per cubic centimeter, the total N concentration being, therefore, 2.46 mg. per cubic centimeter. The activity of the mixture was then compared with that of horse serum alone (1.23 mg. N per cubic centimeter), and with that of rabbit serum alone (1.23 mg. N per cubic centimeter). The results are shown in Figure 12. It is clear that the presence of the inactive horse serum had no other effect on the active rabbit serum in the mixture than to lower the viscosity value for the initial drop, a lowering which persisted throughout the entire incubation period during which the rabbit serum was effective. In other words, the mixture of the two sera, twice as concentrated in milligrams of N per cubic centimeter as either of the sera alone, began the incubation period at a more advanced depolymerization state than occurred with either serum alone. The degree of activity of the rabbit serum in the mixture and of the rabbit serum alone was practically identical, as is shown by the respective curves in Figure 12, which are practically parallel.

Rat serum (fig. 11) has little or no depolymerase activity. On the other hand, rat milk (fig. 10) has the greatest activity of all the samples of milk investigated. In order to determine whether the serum

of the rat during the lactation period might reveal some activity not observed in the nonlactating rat, parallel investigations on the sera of lactating and nonlactating rats were conducted. The results obtained, however, in the two cases were practically identical. As a corollary to this investigation, a study was made of the sera of normal C_3H mice and of the sera of C_3H mice with spontaneous mammary tumors. No difference whatever could be observed in the degree of activity of depolymerase in the two samples of sera.

THE EFFECT OF PREHEATING PROTEINS AND SERA BEFORE MIXING WITH THYMONUCLEATE

So far, it has been shown that oxidizing and reducing agents, dialysis, and the mixing of various sera have no observable effect on the activity or nonactivity of specific sera. The number of agents which might be used for this purpose are limited, owing to the fact that materials which might affect the sera would also, unless conveniently removed beforehand, affect the nucleate after mixing. In addition, the limitations imposed by the use of the capillary viscosity method made it necessary to avoid any procedure which might lead to a heterogeneous mixture. However, sera and solutions of serum albumin will remain homogeneous after pro-

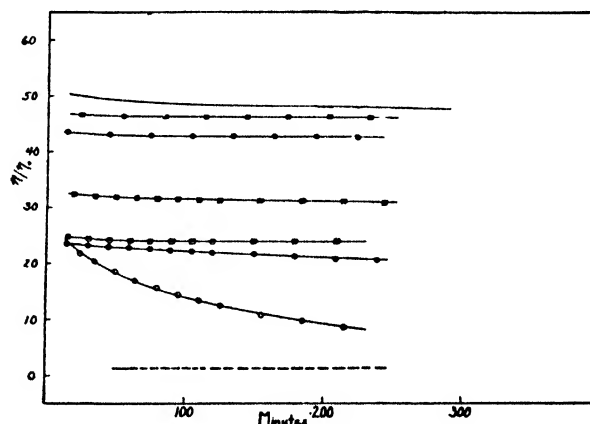


FIG. 13. The incubation of thymonucleate with preheated samples of horse and of rabbit serum. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 1.23 mg. N per cc.

— Control, ○ Rabbit (unheated), ⊗ (preheated 5 hrs.), ● (preheated 15 hrs.), □ Horse (unheated), ⊠ (preheated 5 hrs.), ■ (preheated 15 hrs.), — — — Control

longed heating at 60–63°, and it was determined to investigate the effect of preheating sera before mixing with thymonucleate.

When rabbit serum was heated at 60–63° for five hours, then cooled and mixed with thymonucleate, it lost the depolymerase activity although it still retained the effect of causing the initial drop in the viscosity of the mixture. This is clearly revealed in

Figure 13. When, however, the rabbit serum was heated at this temperature for 15 hours, not only was the depolymerase activity gone, *but it had lost most of its effect in causing the initial drop in viscosity* (fig. 13). Heating of the serum for 40 hours before mixing with thymonucleate had very little more effect than that elicited by the 15 hours' period. Horse serum preheated at 60-63° for five hours before mixing with nucleate lost some of its ability to cause a drop in the viscosity of the mixture; after 15 hours at this temperature this ability had nearly disappeared (fig. 13). In all cases, the protein content of the sera was the same after heating as before (amount precipitated by five percent trichloroacetic acid).

The results obtained with heated sera are highly significant. Apart from the fact that they suggest that the depolymerase activity is destroyed by heat, they reveal that the ability of the proteins of the sera to lower the viscosity of thymonucleate is dependent upon the native unaltered state of these proteins. When the proteins are denatured by the prolonged standing at 60-63°, the viscosity-lowering capacity, i.e., the depolymerizing capacity, is nearly destroyed. This capacity of the serum proteins is apparently a function of the native structure of the proteins and is not, primarily at least, a function of the kind of chemical groupings in the protein. These groupings, after all, are present both in heated and unheated sera.

A similar series of experiments with horse serum albumin revealed that a much longer period of heating solutions of this purified protein is necessary to destroy the capacity for viscosity lowering than is necessary for the whole serum. In order to attain the same results shown by serum at 15 hours, it was necessary to heat the albumin solution for about 60 hours. This suggests that the complex mixture of proteins in the serum is more labile toward heat than is a single protein. Egg albumin could not be studied under these conditions because it quickly coagulates.

These results have an important bearing on the behavior of nucleoproteins *in vitro*. They strongly suggest that nucleic acid will not be affected by nor combine with a denatured protein, although it will be greatly affected by combination with any native protein. On the other hand, we may draw the implication from these results that when the protein part of a nucleoprotein is denatured, that the nucleic acid will be released. This implication is supported by all experience on the isolation of nucleic acid from tissues, for it has invariably been necessary to denature completely all the proteins of the tissues in order to isolate the nucleic acid in the native, unaltered state. What this further implies is that the depolymerization of nucleic acid, effected by the native protein, is reversed when the protein is denatured. The evidence for this assumption may be illustrated by the results above of the present authors (1940) on the nucleoprotein of liver. This

conjugated protein does not exhibit double refraction of flow nor structural viscosity. When it is denatured, the nucleic acid split from it is highly viscous and doubly-refracting during flow. The present authors have also shown that thymonucleate depolymerized to such an extent by salt that the streaming birefringence and structural viscosity have disappeared will have these properties nearly completely restored

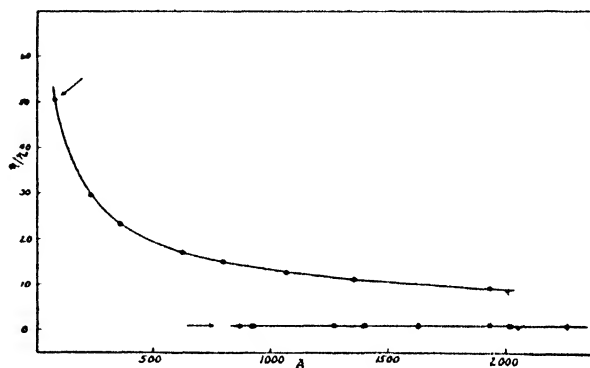


FIG. 14. The viscosity of mixtures of thymonucleate with various fluids as a function of the velocity gradient. The arrows indicate data at 16 cm. water pressure.

● Control, ○ Rat Milk, ◇ Rabbit serum, □ Mammary tumor

after physical removal of the salt (Greenstein and Jenrette, 1940). It is possible to speculate, therefore, on the rapid formation and breakdown of nucleoproteins induced by alternating and reversible native and denatured states of the tissue proteins concerned. However, further experimental work is needed to fill in this picture.

THE STRUCTURAL VISCOSITY OF CERTAIN MIXTURES AFTER ATTAINMENT OF CONSTANT VISCOSITY

Although the various agents (extracts, proteins, etc.) lowered the viscosity of mixtures with thymonucleate, the determination of the viscosity was performed at a single pressure. In order to ascertain whether the structural character of the viscosity had likewise diminished or had been altogether destroyed, it is necessary to determine the viscosity at different pressures.

When the viscosity value at 16 cm. H₂O had reached a constant level for mixtures with thymonucleate of rat milk, rabbit serum, and mammary tumor extract, the pressures were varied and the viscosities determined at the new pressures for each mixture. Since the viscosity of these mixtures is little more than that of water, the range of pressures available is rather limited, but it is clear from Figure 14 that *the viscosities are independent of the velocity gradient employed*. The mixtures have, therefore, lost the property of anomalous viscosity, a property originally belonging to the thymonucleate component of the mixture (control in fig. 14). The

streaming birefringence also characteristic of the nucleate component had also disappeared. The thymonucleate at this stage may, therefore, be said to be completely, or nearly completely, depolymerized.

NOTE ADDED IN PROOF

A sample of crystalline ribonuclease, kindly donated by Dr. Kunitz, which rapidly depolymerized yeast nucleic acid, produced no enzymatic depolymerization whatever of thymonucleic acid at as high a concentration as 8 mg. of protein per cc. This result is all the more striking since we have recently found intense thymonucleodepolymerase activity in beef pancreas, the tissue from which ribonuclease has been isolated. It is probable that the depolymerization of each type of nucleic acid requires a separate enzyme.

Supplementing these observations, we have recently found that several plant embryos derived from the sunflower, corn, wheat, lima bean, and pumpkin, all possess marked thymonucleodepolymerase activity. This finding fits in well with the discovery of thymonucleic acid in plant nuclei (Feulgen, R., Behrens, M., and Mahdihassan, S., 1937, *Z. physiol. Chem.* 246:203), and lends support to the contemporary view that no distinction should be drawn between "plant" and "animal" nucleic acid.

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DISCUSSION

MAZIA: Would you anticipate that in your system nucleoproteins would not have stream birefringence and structural viscosity?

GREENSTEIN: Many do not.

MAZIA: But some do and in those cases it is easy to form gels.

GREENSTEIN: But a gel of thymonucleohistone does not show stream birefringence.

MAZIA: What is the viscosity behavior of a solution of thymonucleohistone?

GREENSTEIN: You must go to such dilute solutions that you cannot pick up the structural effect.

MAZIA: You can get fairly high concentrations in solution, can't you?

GREENSTEIN: But how are you going to make capillary viscosity measurements with a gel? If the solution is too dilute, you cannot pick it up with this method.

DELBRÜCK: Is the depolymerization effect of a salt reversible when the salt is dialyzed off?

GREENSTEIN: The streaming birefringence returns in nearly the original intensity.

SCHULTZ: Does this apply in ultraviolet irradiation also or is it reversible?

GREENSTEIN: It has not been found as yet to be reversible in this case.

MIRSKY: How high is the molecular weight of these aggregates?

GREENSTEIN: At a definite limited concentration, sodium thymonucleate gives sharp boundaries, with

molecular weight of several million. This figure because of the viscosity expression is necessarily approximate.

MIRSKY: What is the concentration of nucleic acid?

GREENSTEIN: About .2 percent.

MIRSKY: What happens when it is diluted; if it is a rod-shaped particle, the sedimentation constant should change when you dilute it.

GREENSTEIN: You cannot get good sedimentation beyond a certain point.

FANKUCHEN: Can you restore the original flow birefringence?

GREENSTEIN: Yes, by dialyzing the salt or by precipitating out the material and washing.

FANKUCHEN: Could you then get larger particles instead of smaller ones?

GREENSTEIN: It is not likely since the two properties decrease together in parallel fashion. Ultracentrifugal measurements show a considerable drop in molecular weight.

FANKUCHEN: There must also be a drop in viscosity.

GREENSTEIN: Yes.

MAZIA: Can you not answer this by studying the viscosity at high pressures relative to low pressures? Would there be the same viscosity change?

GREENSTEIN: No, there is a larger deviation for low pressures than for high pressures.

ANSON: What happens if a nucleic acid solution is heated?

GREENSTEIN: The thymonucleate decreases in viscosity and intensity of birefringence.

MULLER: Is this reversible?

GREENSTEIN: It is to some extent by cooling.

STANLEY: There is a certain amount of parallelism between the experiments Dr. Greenstein has described and the behavior of tobacco mosaic virus. If material such as urea, guanidine, etc., be added to the virus, it breaks up into small particles, and the reaction appears to be irreversible. The disintegration of the virus was followed by means of osmotic pressure and sedimentation measurements. The disintegration is accompanied by a decrease in viscosity and by loss of stream birefringence. I should like to ask if the initial drop followed by a levelling off, shown in your figures, might represent a difference in rate, but perhaps not a difference in kind.

GREENSTEIN: Why should an enzyme suddenly come down to this level and stay there instead of depolymerizing all the way?

STANLEY: The difference between the two may be due to some reaction between an inhibitor and the enzyme.

GREENSTEIN: We tried preheating the material but the curves are still somewhat flat.

SCHULTZ: What happens to these properties when you add basic proteins?

GREENSTEIN: You get a precipitate so this is not useful for these experiments.

SCHULTZ: There are two factors here, a change in the ratio of nucleic acid to protein, and the kind of protein present. During the cell cycle the state of aggregation of nucleic acid changes; if you take the salivary gland nucleus, which is in the interphase, the nucleic acid in the bands is not highly polymerized and shows no birefringence. In interphase chromosomes, according to Caspersson, there is a different type of protein than in the mitotic chromosomes. He has taken over the Kossel cycle and applied it to mitosis, according to which the ratio of protein to nucleic acid changes so that at metaphase you have the maximum amount of nucleic acid. This is exactly the type of behavior of protein that would give you changes of polymerization of nucleic acid in the direction observed but this picture leaves out any reversible denaturation.

GREENSTEIN: This is the essence of the scheme. While it is true that myosin and the tobacco mosaic virus undergo irreversible denaturation, it is likewise true that globular proteins are capable of a reversible denaturation.

UBER: In reducing the viscosity with ultraviolet radiation, what is the quantum yield?

HOLLAENDER: To reduce the structural viscosity in .5 percent solution by 10 percent, 5600 quanta per molecule are needed at λ 2537 Å. One million is taken as the molecular weight. The calculation of energy is difficult since the material has a very high viscosity, but the value gives the order of magnitude. A 10 percent reduction in viscosity may mean a breaking down of the molecule into several fragments. This is very tentative.

FANKUCHEN: Can you restore the stream birefringence, and if so, can you decide whether you are breaking the particles up or not?

GREENSTEIN: The conditions for reversing the radiation effect have not as yet been found.

FANKUCHEN: Doesn't this mean that you have something different happening here? You would expect the same amount of irreversibility in both cases.

GREENSTEIN: I don't see why necessarily. We probably haven't found all the conditions of reversibility as yet.

FANKUCHEN: You are fairly certain that this is depolymerization rather than aggregation?

GREENSTEIN: We have preliminary data on molecular weight that indicate this.

UBER: Have you plotted the viscosity behavior with ultraviolet as a function of dosage? What type of curve represents the reaction?

HOLLAENDER: We have irradiated bacteria in the same suspension with the sodium thymonucleate. We get a killing curve for bacteria and an action curve for the changes in structural viscosity of the same shape. But you should not pay much attention to the shape of the curve until we get more data.

DELBRÜCK: There is a doubt in my mind whether your interpretation of the viscosity changes is cor-

rect in the case of the protein effect. When the relative viscosity is measured, do you measure the viscosity of the protein solution and compare it with the viscosity of the protein solution plus nucleic acid? To interpret this, a complication comes in from the interaction between the two, independent of any depolymerization. How can you tell that nucleic acid is depolymerized when you add something which by itself alters the viscosity?

GREENSTEIN: The contribution of the protein to the viscosity of the mixture is almost negligible compared with that of the nucleic acid.

DELBRÜCK: You might have any kind of interaction.

GREENSTEIN: It is the magnitude of the changes which I would like to emphasize. The drop is too great for simple electrostatic interaction.

DELBRÜCK: Don't you get enormous changes in viscosity when you change from tactoid to normal solution?

STANLEY: Is there an explanation for the difference in ease of depolymerization of yeast nucleic acid and thymus nucleic acid?

GREENSTEIN: I cannot say.

THE CHEMICAL COMPOSITION OF STRAINS OF TOBACCO MOSAIC VIRUS

W. M. STANLEY AND C. A. KNIGHT

There is a striking similarity between the properties that have been found for the viruses that have been isolated in the form of high molecular weight nucleoproteins and the properties that have been ascribed to genes (Muller, 1935; Demerec, 1939; Stanley, 1940). As may be seen from Figure 1, one estimate of the approximate size of a gene would place it in the midst of the viruses, many of which are larger and many of which are smaller. Both viruses and genes may be regarded as large nucleoproteins that have the ability to perpetuate themselves by reproduction within, and only within, certain specific living cells. Both may undergo sudden changes either spontaneously or as a result of irradiation, and these changes are then faithfully reproduced in subsequent generations. Although it has not proved possible to isolate and study genes *in vitro* without loss of their viability, in the case of the viruses it is possible to remove them from their host cells, obtain them in pure form, and subject them to extensive study *in vitro* without impairment of their peculiar activity. In addition, it is possible to change the structure of a virus *in vitro* by chemical means and then determine the effect of the change by inoculating a host with the altered virus (Anson and Stanley, 1941; Miller and Stanley, 1941). Although the points of similarity between viruses and genes may be fortuitous, it nevertheless seems quite likely that a study of viruses will provide much information pertinent to the question of the nature of genes and of their mode of action.

The fact that genes are usually duplicated but once during each cell division, whereas a virus particle is duplicated many, many times within a single cell, may appear to represent a point of difference, yet this difference is in reality essentially one of degree. It is well known that more than a single doubling of chromosomes occurs in some cells, and the action of colchicine in inducing polyploidy in certain plant cells is good evidence that the basic mechanism of gene duplication may be caused to continue to a certain extent within a single cell (Blakeslee, 1939). In the case of viruses, it is known that the concentration and hence the extent of duplication of a given virus may vary over a hundredfold, depending upon the type of the host. Some viruses appear to exist in such a low concentration within cells that it has not proved possible to isolate them. It is obvious, therefore, that, although genes tend to multiply but once within a given cell whereas viruses multiply more than once, environmental conditions may increase markedly the number of duplications of the former and

decrease markedly the number of duplications of the latter.

It has been known for some years that large, thick, hexagonally-shaped plates occur in the cytoplasm of mosaic-diseased cells of Turkish tobacco plants. Recent data indicate that these crystals are composed almost exclusively of tobacco mosaic virus and, hence, that there is a very high concentration of virus in the cytoplasm of these cells (Beale, 1937). The fact that tobacco mosaic and other viruses reach a high concentration in the cytoplasm of cells, whereas genes are contained within the nuclei of cells, appears to be of no special importance, for some viruses appear to reach a high concentration within the nuclei of the cells. In the case of severe etch virus, large crystalline inclusions appear in the nucleus and not in the cytoplasm of the diseased cell (Kassanis, 1939). However, it must be admitted that at present it is not known whether viruses are synthesized within the cytoplasm, within the nucleus, or at the interphase between the two.

About 20 viruses have been purified and, so far as tested, they have, with one exception, failed to give a test for desoxy sugar but have given tests for pentose and hence probably contain nucleic acids of the yeast type. Purified preparations of the elementary bodies of vaccinia have been found to give a test characteristic of thymus nucleic acid. Similar results have been obtained with psittacosis virus. Genes are usually considered to be made up of nucleoprotein containing the thymus or desoxyribose type of nucleic acid. However, many cells are known to contain both types of nucleic acid and, even if additional work should confirm the fact that genes contain only desoxyribonucleic acid, it could hardly provide an important point of difference between viruses and genes. If genes may be considered to be represented by the nucleoprotein isolated from fish sperm, they would appear to contain about 50 percent of desoxyribose nucleic acid rather loosely bound to about 50 percent of protein of the very basic histone and protamine types. Viruses, on the other hand, have been found to contain from 5 to about 40 percent of ribonucleic acid. In general, the nucleic acid appears to be firmly bound and is not dissociated by the addition of salt but requires the action of rather strong alkali for separation from the protein component. However, a portion of the 40 percent of nucleic acid contained in tobacco ringspot virus may be removed by salt dissociation. It may prove of some importance to recognize that there may be several quite different types of linkages between nucleic acid and protein. Furthermore, with time, the presence in viruses and genes of nu-

cleic acids differing slightly in structure may be established. Loring has, for example, already obtained evidence that the uridylic acid isolated from tobacco mosaic virus may be isomeric rather than identical with that from yeast nucleic acid (Loring, 1939). There is as yet no evidence that the

COMPARATIVE SIZES OF VIRUSES

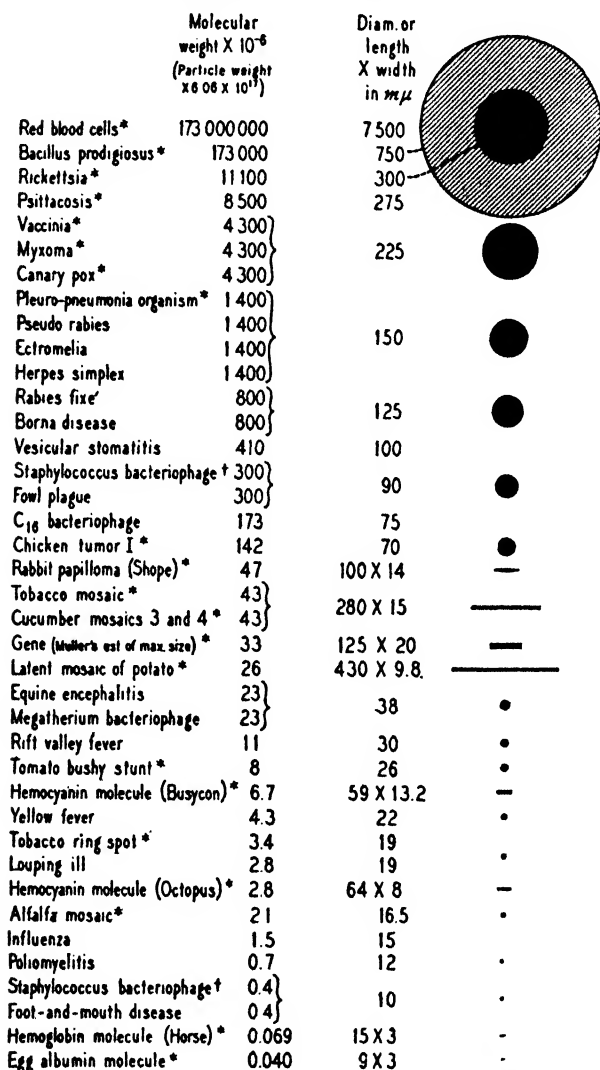


FIG. 1. A chart showing the relative sizes of several selected viruses, including bacteriophages, as compared to those of red blood cells, *Bacillus prodigiosus*, rickettsia, pleuropneumonia organism, and protein molecules. The figures for size have been arbitrarily selected from data available in the literature. Particles known to be asymmetric are so indicated and the estimated length and width and the molecular weight in accordance with the asymmetry are given. In other cases where the particles are known or assumed to be spherical, the diameter and the molecular weight based on a sphere of density 1.3 are given. * = evidence regarding shape available. † = large size from filtration and sedimentation of concentrated solutions and small size from diffusion of dilute solutions.

protein components of the viruses are similar to the protein component of sperm nucleoprotein in containing a high percentage of basic amino acids. Tobacco mosaic virus, which has been studied in greater detail than any other virus, has been found to contain but 9.0 percent arginine. Histidine appears to be absent and, despite the fact that the majority of the amino acids of the protein component have been accounted for, no other basic amino acid has been found in large amount. If it can be demonstrated that genes are made up of the protamine and histone type of protein, this might prove to be a point of difference between viruses and genes. However, at the present time the points of similarity between viruses and genes appear to be sufficient to warrant an examination of some recent information on the chemical composition of variant strains of tobacco mosaic virus as a possible means of learning something of the nature of gene mutation.

Following the isolation of tobacco mosaic virus in the form of a protein of unusually high molecular weight and the subsequent characterization of the virus as a nucleoprotein possessing quite definite properties and composition, it became of interest to determine the nature of materials isolated from plants diseased with strains of this virus. It was well known that a given virus usually existed in the form of different, although usually immunologically related, strains which were capable of causing somewhat similar yet different diseases. Although some were of high virulence and others of low virulence, many examples were known of the conversion of one strain into another strain either spontaneously or as a result of passage in an unnatural host; hence, it seemed reasonable to assume a common origin for the strains of a given virus. The case first examined from a chemical standpoint was that of the yellow aucuba strain of tobacco mosaic virus, and it was soon established that plants diseased with this virus strain contained a high molecular weight nucleoprotein, the general properties of which were very similar to those of ordinary tobacco mosaic virus nucleoprotein (Stanley, 1937; Bawden and Pirie, 1937). However, small differences have been found so that, despite the great similarity in the general properties of the two nucleoproteins, it is possible to mix the two and then separate them again into two fractions, one carrying the tobacco mosaic virus activity and the other the aucuba mosaic virus activity. Nevertheless, practically nothing is known concerning the exact difference in the chemical structure which is responsible for the difference between aucuba and tobacco mosaic viruses, despite the fact that there is good reason to believe that the aucuba strain arises directly from the ordinary strain of tobacco mosaic virus. It is obvious that such a transition, that is, the mutation of a virus or also of a gene, must be attended by changes in the chemical structure. It is possible that such changes might occur in the nucleic acid or the

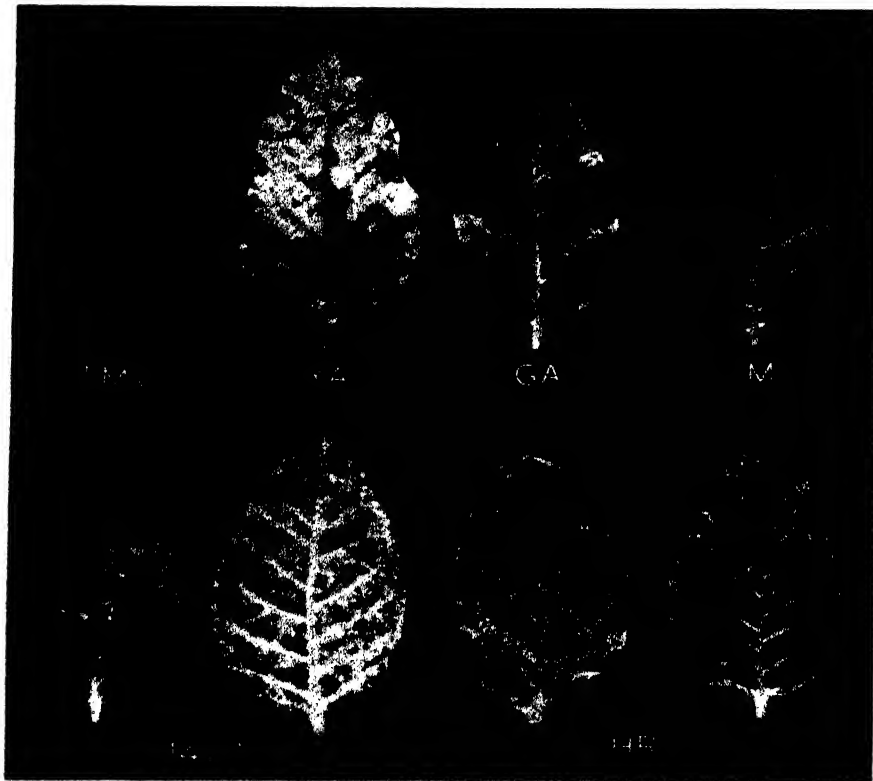


FIG. 2. Leaves of Turkish tobacco plants showing symptoms typical for each of 6 strains of tobacco mosaic virus. TMV = tobacco mosaic virus; YA = yellow aucuba; GA = green aucuba; M = Holmes' masked strain; J14D1 = a derivative, isolated by Dr. L. O. Kunkel, of Dr. J. H. Jensen's J14 virus; and HR = Holmes' ribgrass strain. The leaf showing the masked strain (M) is practically indistinguishable from a normal leaf, although it contains an appreciable amount of virus. The J14D1 and HR viruses differ from the other strains shown in giving distinct primary lesions on the inoculated leaves as well as typical secondary symptoms. In each of these cases, the first leaf shows characteristic primary lesions and the second leaf the secondary symptoms. (Photograph by J. A. Carlile.)

protein components or in both. Recently, X-ray and gamma ray induced mutations of tobacco mosaic virus have been reported (Pfankuch, Kausche and Stubbe, 1940). On the basis of a 15 percent difference between the phosphorus contents of the original strain and one of the altered strains, presumably established by only two analyses in each case, it was concluded that such mutations could be attributed to irradiation-induced alterations in the nucleic acid part of the virus molecule. In contrast to these findings, data are given in the present paper which show that, in the case of spontaneously arising strains of tobacco mosaic virus, there are definite and quite characteristic differences in the amino acid composition of certain strains and that the chemical differences between strains probably lie not in the nucleic acid but rather in the protein part of the virus molecule.

Preliminary to a more extensive investigation, analyses were made for tyrosine, tryptophane, and phenylalanine in six well characterized and distinctive strains of tobacco mosaic virus and in the related cucumber viruses 3 and 4. Representative leaves from Turkish tobacco plants diseased with the six strains of tobacco mosaic virus are shown in Figure 2. The choice of determinations of the amounts of the aromatic amino acids for an approach to this study was prompted by the close association of biological activity in certain enzymes and hormones with the integrity of tyrosine. Moreover, evidence has accumulated which emphasizes the importance of aromatic nuclei in serological specificity (Landsteiner, 1936). Since relationships between viruses and between virus strains are partly established by serological tests, any chemical evidence which can be correlated with such tests becomes especially desirable.

AMINO ACID ANALYSES

Purified virus preparations obtained from filtered infectious juices by differential centrifugation were dialyzed against flowing distilled water for 48 hours, frozen and dried *in vacuo*, and then further dried to constant weight at 110° C. in a drying oven. The white fluffy material thus obtained was used for amino acid and phosphorus analyses.

Tyrosine was determined by the Bernhart micro-method (1938), tryptophane by the Shaw and McFarlane glyoxylic acid procedure (1938), and phenylalanine by Block's modification of the Kapeller-Adler reaction (Block, 1938; Block, Jervis, Bolling and Webb, 1940). Details concerning the application of these methods are given elsewhere (Knight and Stanley, 1941). The results of individual analyses showed a maximum deviation from the averages listed in Table 1 of ± 0.1 percent for the tyrosine and ± 0.2 percent for the tryptophane and phenylalanine values. Analysis of 12 preparations of tobacco mosaic virus indicated the presence of 3.8, 4.5, and 6.0 percent of tyrosine, tryptophane, and phenylalanine, respectively. These values agree

quite well with those recently reported for tobacco mosaic virus (Ross, 1941). The results obtained for yellow aucuba, green aucuba, Holmes' masked, and J14D1 strains of tobacco mosaic virus were similar. The small differences observed may or may not prove significant, since they are within the experimental error. However, analyses of these strains for other amino acids may, of course, show differences sufficiently large to account for the differences in the biological properties of these strains. Pronounced

TABLE 1. AROMATIC AMINO ACIDS AND PHOSPHORUS IN STRAINS OF TOBACCO MOSAIC VIRUS AND IN CUCUMBER VIRUSES 3 AND 4

Virus	No. of preparations	Tyrosine p.c.	Tryptophane p.c.	Phenylalanine p.c.	P p.c.
Tobacco mosaic	12*	3.8	4.5	6.0	0.56†
Yellow aucuba	3	3.9	4.2	6.3	0.52
Green aucuba	2	3.9	4.2	6.1	0.54
Holmes' ribgrass	4	6.4	3.5	4.3	0.53
Holmes' masked	2	3.9	4.3	6.1	0.54
J14D1	2	3.8	4.4	6.1	0.55
Cucumber virus 4	7	3.8	1.4	10.2	0.54
Cucumber virus 3	1	4.0	1.5	10.0	0.56

* Two or more analyses were made on each preparation. The results of individual analyses showed a maximum deviation from the averages listed of ± 0.1 percent for the tyrosine and ± 0.2 percent for the tryptophane and phenylalanine values.

† The values in this column represent the average of duplicate determinations on two or more preparations, with the exception of cucumber virus 3. Almost all of the results of individual analyses were within the range 0.52 to 0.58 percent.

differences were found in the case of Holmes' ribgrass strain (Holmes, 1941) and cucumber viruses 3 and 4. Tyrosine, tryptophane, and phenylalanine contents of 6.4, 3.5, and 4.3 percent, respectively, were found for the ribgrass strain, while similar analyses of 7 preparations of cucumber virus 4 and one preparation of cucumber virus 3 indicated the presence of about 3.8, 1.4, and 10.2 percent of tyrosine, tryptophane, and phenylalanine, respectively.

PHOSPHORUS ANALYSES

Phosphorus determinations were made on 5-10 mg. samples of dried virus according to the method of King (1932). Individual analyses for phosphorus in samples of the various strains resulted in values ranging from 0.5 to 0.6 percent, with a majority of the values falling within a somewhat narrower range. The differences between the average phosphorus value for tobacco mosaic virus and the phosphorus values for any of the strains listed in Table 1 are smaller in many cases than the differences between individual samples of tobacco mosaic virus or even between values obtained during repeated analyses of the same sample. Since the amount of phosphorus in a nucleoprotein is indicative of the quantity of nucleic acid present, it may be concluded that there is no demonstrable quantitative

difference between the nucleic acid components of the viruses examined. This does not exclude the possibility of qualitative dissimilarities among the nucleic acid constituents. However, such differences, if present, are probably small, since the positive pentose and negative desoxypentose tests which were obtained for each virus indicate that all of them, as has been definitely shown for tobacco mosaic virus (Loring, 1939), contain the yeast type of nucleic acid.

SEROLOGICAL REACTIONS

Preparations of the viruses used in serological tests were made by purely physical methods involving filtration and differential centrifugation of the juices from infected Turkish tobacco plants, or from cucumber plants in the cases of cucumber vir-

from the composition of tobacco mosaic virus were apparent. It may or may not be significant that in all cases the total percentages of aromatic amino acids were about the same.

DISCUSSION

The capacity of one strain of a virus to protect a fully infected plant from further infection by a second strain and the ability of one strain to react with the antiserum of another are among the most important characteristics by means of which plant virus strains are recognized. In addition, viruses shown to be related by plant protection and serological tests invariably possess similar physical-chemical properties and are transmitted by the same methods. By these criteria, the viruses discussed in the pres-

TABLE 2. PRECIPITATION OF STRAINS OF TOBACCO MOSAIC VIRUS AND CUCUMBER VIRUSES 3 AND 4 WITH TOBACCO MOSAIC VIRUS ANTISERUM

Antigen	Dilution of antigen (1/1 = 1 mg. per ml.)					
	1/1	1/4	1/16	1/64	1/256	1/1024
Tobacco mosaic	++++	++++	+++	++	+	±
Yellow aucuba	++++	++++	+++	++	+	—
Green aucuba	++++	++++	+++	++	+	—
Holmes' masked	++++	++++	+++	++	+	—
Holmes' ribgrass	—	+	++	+	—	—
J14D1	++++	++++	+++	++	+	—
Cucumber virus 4	—	+	+	+	—	—
Cucumber virus 3	—	+	+	+	—	—

+ Signs indicate the degree of precipitation.

uses 3 and 4. Tobacco mosaic virus antiserum was obtained from the blood of a rabbit 8-10 days after the last of 5 spaced intravenous injections of a total of about 40 mg. of virus. Precipitin tests were made by adding 0.3 ml. of antigen at various dilutions to tubes containing 0.3 ml. of tobacco mosaic virus antiserum diluted 1:10. All dilutions were made with 0.85 percent sodium chloride. After mixing, the tubes were incubated at 37° C. for 2 hours, placed in a refrigerator overnight, and examined for precipitates.

Precipitin tests with strains of tobacco mosaic virus and cucumber viruses 3 and 4 as antigens and tobacco mosaic virus antiserum demonstrated a strong serological relationship between tobacco mosaic virus and all of the viruses tested, with the exception of Holmes' ribgrass strain and cucumber viruses 3 and 4 (see Table 2).

A comparison of the amino acid values for the viruses examined (table 1) with the results of the serological tests reveals a striking similarity in the outcome of the two types of experiments. Within the limits of accuracy of the methods employed, the aromatic amino acid compositions of the viruses examined were very close to that of tobacco mosaic virus, with the exception of Holmes' ribgrass strain of tobacco mosaic virus and cucumber viruses 3 and 4. In each of the latter cases pronounced differences

ent paper are strains of tobacco mosaic virus, and hence may be presumed to have had a common origin. They are known to possess very similar physical-chemical properties, are transmitted in the same manner, react with each other's antiserum, and plants fully infected with one are protected against infection from another. In the cases of cucumber viruses 3 and 4, it has not been possible to apply the plant protection test, for no host common to the cucumber viruses and tobacco mosaic virus has thus far been found. The cucumber viruses are somewhat unusual in that they have been found to multiply only in members of the Cucurbitaceae. Despite this fact, it is commonly believed that cucumber viruses 3 and 4 may have arisen from tobacco mosaic virus through some fortuitous event. In this connection it is important to note that these nucleoproteins have very similar general properties (Bawden and Pirie, 1937; Knight and Stanley, in press), and especially that among a large number of viruses cucumber viruses 3 and 4, tobacco necrosis virus, and the viruses of the tobacco mosaic group stand alone in their remarkable ability to resist heat. Therefore, the pronounced differences between the amounts of aromatic amino acids found in tobacco mosaic virus, Holmes' ribgrass strain, and cucumber viruses 3 and 4 demonstrate clearly for the first time the chemical nature of certain changes

which may accompany the variation of a virus.

The remarkable coincidence of results of the serological tests and the amino acid analyses is of special interest. Extensive work will be required to establish fully the significance of this agreement, but, when considered with evidence of other types pointing in the same direction (Landsteiner, 1936), it appears to reemphasize the role of aromatic amino acids in the serological specificity of proteins.

The differences in composition of tobacco mosaic virus and the Holmes ribgrass virus are particularly important, for the latter is unequivocally a strain of tobacco mosaic virus (Holmes, 1941). In this case at least, the formation of a virus variant obviously has involved fundamental changes in the composition of the protein rather than simpler alterations of the type frequently postulated, such as a shift of double bonds, a gain or loss of certain reactive groups or a rearrangement of basic units to form a new pattern. However, it should be recognized that this gross type of change may not apply to all virus mutations. For example, Dr. Gowen has just presented in this symposium the first extensive data indicating that variants of tobacco mosaic virus may be produced *in vitro* by X-ray treatment. In earlier work, irradiation was not found to cause an increase in the frequency of formation of variants, or the data advanced in support of such a hypothesis were too scanty to enable a definite decision. Either large or small alterations in chemical structure may be postulated as having occurred in the formation of these variants by X-ray treatment, in view of the fact that they have not yet been subjected to chemical analysis. The irradiation may have caused primary changes in vulnerable centers concerned with the reproduction of the virus in such a manner that the subsequent multiplication of irradiated molecules was accompanied by more pronounced secondary changes in structure similar to those demonstrated in the present work on spontaneously arising variants. Since X-ray data (Bernal and Fankuchen, 1941) indicate that the tobacco mosaic virus particle is made up of numerous repeat sub-units having the dimensions $68 \times 88 \times 88$ Å, it is conceivable that such a primary change may have occurred within one of these sub-units and was then subsequently transferred to all other sub-units. It is also possible that irradiation may have caused small alterations in structure which were then exactly duplicated in the reproduction of the virus. Valuable information relative to these possibilities could be obtained by chemical analysis of the strains produced by irradiation. Such analyses would establish the magnitude of the structural change or changes involved. Should these prove to be gross changes, it would immediately suggest that they were of secondary origin. In this connection, it is hoped that experiments of the type reported by Miller and Stanley (1941), using virus in which all of the molecules have been chemically modified, will throw some light on the type of change which the

virus will respond to or reproduce. In view of the marked changes which the present data have shown to occur in the variation of a virus, it seems likely that a new strain has arisen in each case by a diversion of the synthetic process by which the virus multiplies rather than by an alteration of some of the completely formed virus molecules. It is difficult to postulate a simple mechanism by means of which a molecule containing 3.8, 4.5, and 6.0 percent, respectively, of tyrosine, tryptophane, and phenylalanine may be converted directly into a molecule containing 6.4, 3.5, and 4.3 percent, respectively, of these amino acids. Although preliminary results indicate that the particles of the Holmes ribgrass strain of virus have approximately the same size and shape as those of tobacco mosaic virus, the X-ray data (Bernal and Fankuchen, 1941) indicate that the diameter of the particles of the cucumber viruses 3 and 4 is slightly less than that of tobacco mosaic virus. In the latter case, the gross change in amino acid composition is accompanied by a change in the shape of the virus molecule. Other indications of differences in the size and shape of variant strains of tobacco mosaic virus are discussed elsewhere (Stanley and Anderson, 1941). Practically nothing is known of the mechanism by means of which a virus particle is duplicated, and there is at present little basis for selection from such alternatives as longitudinal growth and lateral division, lateral growth and longitudinal division, growth from a point, some cataclysmic event, etc. It does seem unlikely, however, that the deep-seated changes which have been established in the cases of 3 strains could have been caused directly by alterations within completely formed virus molecules. Unless such direct changes can be established experimentally, it appears more reasonable to assume that the 3 strains arose as a result of fortuitous events during the synthesis of the virus. Because of the nature of the present approach in which a sample of virus produced as a result of millions of duplications is examined, it is impossible to determine whether the changes in composition occurred in a single step or as a result of the cumulative effect of a number of successive alterations in structure during the multiplication of the virus. Information regarding this question would become available should it prove possible either by irradiation or by chemical means to cause *in vitro* a given heritable change in a large percentage of the molecules of the sample. So far, the chemical changes that have been achieved have not proved to be heritable and the changes attributed to X-irradiation appear to affect only a very small percentage of the molecules in a given preparation. However, it is to be hoped that further examination of the nature of the structural differences between variant strains of viruses will provide some idea not only of the mechanism by means of which virus particles are duplicated, but also of the manner in which variant strains are produced. Such information would, of

course, provide a background for similar considerations with respect to the reactions of genes.

SUMMARY

Similarities between the properties of virus nucleoproteins and gene nucleoproteins are discussed, and it is concluded that examination of information on the chemical composition of variant strains of tobacco mosaic virus may be of value in a consideration of the nature of gene mutation. Analysis of 12 preparations of tobacco mosaic virus indicated the presence of 3.8, 4.5, and 6.0 percent of tyrosine, tryptophane, and phenylalanine, respectively. The results obtained for yellow aucuba, green aucuba, Holmes' masked, and J14D1 strains of tobacco mosaic virus were the same as those for tobacco mosaic virus within experimental error. Pronounced differences were found in the cases of Holmes' ribgrass strain of tobacco mosaic virus and cucumber viruses 3 and 4, for the tyrosine, tryptophane, and phenylalanine values for the former were 6.4, 3.5, and 4.3 percent, respectively, and about 3.8, 1.4, and 10.2 percent, respectively, for the cucumber viruses. Serological tests were in complete accord with the results of the chemical analyses.

An indication of the absence of significant quantitative differences in the nucleic acid component of the viruses was evident in the close agreement found in analytical values for phosphorus in the various strains. Qualitative tests showed that the nucleic acid in all of the viruses was of the yeast type.

The decided dissimilarities in the protein portions of 3 of the virus strains, as revealed by the results of the amino acid analyses, show that the mutation of tobacco mosaic virus to form a new strain may be accompanied by changes in the amino acid composition of the virus. Because of the nature of these changes, it is suggested that these new virus strains arose by a diversion of the synthetic process by means of which the virus multiplies rather than by an alteration of completely formed virus molecules.

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DISCUSSION

FANKUCHEN: Was there a change in virus activity when its chemical structure was altered?

STANLEY: No, for in the present experiments in which a change in structure was effected by ketene or phenyl isocyanate care was taken to stop the reaction before changes resulting in a loss of activity had occurred.

GREENSTEIN: Would you be willing to assume from these changes that you get as in tryptophane that you had a simple doubling or trebling of the mean molecular weight? That is, are you simply getting a larger unit?

STANLEY: It would be impossible to account for the observed changes on such a basis.

DAVENPORT: How specific is the relation of virus to host? Can a given kind of virus affect more than one species of plant?

STANLEY: Tobacco mosaic virus has an unusually wide host range for it may infect 46 different species of plants, representing 14 widely separated families.

CHILD: When you continue to transfer tobacco mosaic virus in Turkish tobacco, do you always get the change to Aucuba?

STANLEY: Yes, in practically all instances yellow spots will occur on the leaves and this is indicative of the presence of an Aucuba type or yellow virus. Mutation tends to go in definite channels, although occasionally something different occurs, possibly as a result of environmental effects.

CHILD: So you could say that mutation is the direct result of the environmental change you subject the virus to. A direct effect on *Drosophila* sperm is suggested by the effect of nipagin on vestigial and its subsequent transmission through both eggs and sperm.

STANLEY: Environment certainly affects what you get.

MULLER: Do you consider the nipagin effect to be mutation?

CHILD: It is certainly a transmissible change; I

am carrying on breeding tests to determine the number of generations in which the effect can still be seen.

MULLER: Selection of mutants already present may be made by the host, so you cannot always be certain of the directive nature of the occurrence of new mutations.

STANLEY: That is correct; a selection factor is operating all the time, and during propagation the virus may change considerably. That is why, if you grow many strains long enough, you usually end up with ordinary tobacco mosaic virus, for it represents the strain which gets along the best under ordinary conditions.

STERN: Might the chemically altered virus molecule change immediately to wild type in the new host before it starts to reproduce?

STANLEY: This argument might apply to the S-S virus but I doubt that it could apply to the phenyl ureido group. There is reason to believe that the latter stays intact in the cell.

STERN: Does the virus reproduce in the cell juice?

STANLEY: No, tobacco mosaic virus has not been found to reproduce outside of certain living cells.

RIDDLE: You said you made radical substitutions in the protein of the virus, and treated it with alkali. Does experience with the protein of the virus lead you to consider the virus more or less liable to denaturation than is protein of plant and animal origin?

STANLEY: Tobacco mosaic virus as a nucleoprotein is the most stable of all viruses. It is more susceptible than some and less susceptible than other regular proteins to denaturation.

RIDDLE: Does this suggest that the virus represents an early or primitive form of protein?

STANLEY: No, for some viruses are as unstable as some normal constituents of plants.

PLOUGH: Is it possible to show differences within a normal range of growth? Is the expression in the leaf different at different temperatures?

STANLEY: Yes, variants appear to be produced throughout the normal range of growth. The expression is very different. There is one virus that is very stable in the leaf at normal temperatures but if taken out at the same temperature, it is very unstable. If the temperature is lowered it is stable for several hours in vitro.

PLOUGH: In unstable viruses, is the expression different at different temperatures or are they constant?

STANLEY: The unstable viruses are like tobacco mosaic in that there is usually some temperature effect. However, little work has been done on this general problem.

GREENSTEIN: Are the substituted molecules different serologically from the others?

STANLEY: I suspect there are differences but we have not as yet made careful tests on the derivatives.

FANKUCHEN: Have you any ideas on the remark-

ably constant phosphorus content? Might that not be tied up with the fact that the essential architecture of these molecules is the same? So far the only thing in the chemical constitution known to remain unchanged is the nucleic acid content.

STANLEY: We have not proved that the nucleic acid has not changed. Quantitatively it has not, but qualitatively there may be small changes. German workers found a 15 percent difference in the phosphorus content of two strains and therefore considered that the difference between the two strains resided solely in the nucleic acid portions of the molecule. We have not been able to confirm this. Our evidence indicates that the nucleic acid content is the same and that the structural differences between strains lies in the protein components of the viruses.

MULLER: Can you get active virus back again after treatment with antiserum?

STANLEY: That is one of the stock methods of purifying and isolating some types of antibody, as for example with *Pneumococcus*. Chester showed in virus studies that certain virus antibody precipitates could be treated with enzymes, and in one case he recovered the virus and in another he recovered the antibody.

MULLER: So the antibody produces no essential change in the virus?

STANLEY: No demonstrable change is produced.

RITTENBERG: Can you isolate from normal plants any material similar in properties to the virus nucleoproteins?

STANLEY: We have tried to isolate material similar to the virus nucleoproteins from normal plants. It is possible to get out something when the work is carried out in the cold and such material may have a fairly high sedimentation constant. However the phosphorus content of the material is very low, usually less than .01 percent. So I doubt if it is a nucleoprotein. The properties are quite different from those of the virus nucleoproteins so far isolated.

DAVENPORT: The chemically altered virus in the tobacco cell reproduces the original type of virus. You put the altered molecules into the host and get thousands of particles from it; has not then the cytoplasm contributed to the making of these particles; does the cell cytoplasm not contribute to the restoration of the original type?

STANLEY: That appears to be what happens.

HUSKINS: Is there indication of difference in aggregates formed by chemically different strains?

STANLEY: The strains are so different that they can be mixed and separated by chemical means.

HUSKINS: Is it possible that we have three different categories of change, as in the genetic changes of point mutation, chromosomal aberration and ploidy?

STANLEY: There may of course be different types of changes and I am in favor of the geneticist drawing as many analogies as possible.

THORNBERRY: Are we to assume that all infective particles of the virus are acetylated in the treatment?

STANLEY: If all the molecules were not acetylated, it would be possible to demonstrate this fact by electrophoresis experiments. These experiments indicate the preparations are acetylated uniformly.

FANKUCHEN: Does not infectivity do this?

STANLEY: Yes, that would be another approach.

ANSON: You might describe the experiment with single lesions.

STANLEY: If a few molecules were unchanged and if you plated out the material on a leaf giving a local lesion response, and then used single lesion isolates, you might be able to pick up any difference.

THORNBERRY: Was there a reduction in the number of local lesions?

STANLEY: No, we used fully active virus.

DELBRÜCK: Did the variants arise under laboratory conditions or were they picked up here and there?

STANLEY: Both. The two variants which show differences in their amino acid content were picked up in the field.

DELBRÜCK: Can the two cases outside the laboratory have arisen by step mutation or by a single change?

STANLEY: One cannot be sure. However, I think that there is good evidence that they arose from tobacco mosaic virus. We did not conduct the isolation tests ourselves. It can be shown by many tests (immunology or plant protection, serology, etc.) that there are many tobacco mosaic virus strains in nature.

SCHULTZ: Is there evidence that virus which has been reversibly inactivated is slightly different when you get it back?

STANLEY: There is no evidence that the virus is altered by virtue of inactivation and reactivation. I might mention that no virus has been reversibly denatured as yet.

WEBBER: In your attempts to modify permanently the virus molecule by simple chemical changes have you tried reacting simple diazotized compounds with the tyrosine of the protein? Since it has been shown that at least one type of protein specificity

(serological) is involved at this site, we might expect other types of specific effects by this type of reaction. If this has been done, what influence has it had on the antigen-antibody reaction?

STANLEY: The experiments mentioned are under way. The antigen-antibody results are not yet completed.

DAVENPORT: What is the evidence of the electron microscope on antibodies?

STANLEY: Dr. Anderson and I have studied the reaction between tobacco mosaic virus and its rabbit antiserum by means of the electron microscope and the results have just appeared in the May number of the *JOURNAL OF BIOLOGICAL CHEMISTRY*.

HUSKINS: Has there been a comparative study of the results of X-raying under all sorts of conditions?

STANLEY: Dr. Gowen has done some work along this line, and other methods have been used by German workers.

GREENSTEIN: When the nucleic acid component is removed by alkali, what happens to the protein?

STANLEY: The protein component is broken up into material of lowered molecular weight and is denatured.

SPARROW: Do the viruses reproduce as whole molecules or are they built up by adding the subunits one at a time?

STANLEY: There is only speculation, and no good evidence for any particular method of reproduction.

FANKUCHEN: You never find intermediate steps of virus formation, though.

STANLEY: When preparations of purified tobacco mosaic virus are examined by means of the electron microscope, small particles may be seen together with molecules of the normal size. Dr. Anderson has noted that as the film dries during preparation of the mount, evaporation of water causes a thrashing around which may break the big particles. The small particles should always be in the supernatant liquid following ultracentrifugation if they are really present in the virus preparation. Since they have not been demonstrated in such supernatant liquid it appears that they are absent.

MULLER: Intermediate steps in virus formation must however occur in the cell.

STANLEY: I think so.

PARTICULATE COMPONENTS OF CYTOPLASM

ALBERT CLAUDE

Microscopic and sub-microscopic granules of uniform chemical constitution can be separated from the other components of the cell by submitting tissue extracts to a process of differential centrifugation at high speed (Claude, 1938 a and b, 1939, 1940; Stern and Duran-Reynals, 1939; Stern and Kirschbaum, 1939; Stern, 1939; Kabat and Furth, 1940; Henle and Chambers, 1940). These particulate elements appear to be constant constituents of cells. They have been obtained from many different tissues and no tissue has been examined, so far, which failed to contain them. The problem of interest at the moment is whether these organic granules exist as such in the living cell and what is their chemical constitution. On the ground of physical and chemical analogy, it was suggested recently (Claude, 1940) that these formed elements might represent well known cellular constituents, i.e., mitochondria, or fragments of mitochondria. In the present paper, I should like to bring together a number of observations which appear to support this view.

Separation of tissue components by mechanical means may present an entirely different problem depending on the resistance which the cell and nuclear membranes may offer to disintegration. In the liver, the cell membrane appears to be extremely fragile while the nuclear membrane is remarkably strong. Passing the liver through a meat grinder or a sieve and suspending the pulp in salt solution or water is sufficient to break up the cell membrane of the hepatic cell, thus releasing the cytoplasmic components. On the other hand, the nucleus is not injured by the treatment, and nuclei, apparently intact and showing one or several distinct nucleoli, are set free in the medium. Cell and nuclear membranes appear to be equally brittle in the Brown-Pearce tumor where simple trituration of the tissue destroys the cell membrane and causes fragmentation of the nucleus. Other tissues may resist disintegration unless sand be added. Grinding with sand for three minutes will destroy both the cellular and the nuclear membranes of a lymphoid cell, including the lymphoid cells of mouse and rat leukemia. Grinding chicken tumor I tissue with sand for the same or even for a longer period, and subsequent extraction with buffer solution or water, may leave the cell membrane apparently intact with the nucleus well preserved, as shown by its staining properties. In this case, however, the cytoplasmic area often appears empty, showing that extraction of the visible components has nevertheless taken place. The cell membrane of yeast is unusually resistant and grinding for 30 minutes with an equal weight of beach sand leaves the cell membrane practically untouched. Fresh

yeast cells can be lacerated, however, by using the sharp edge of small fragments of flint, the size of these fragments being about five times the diameter of the yeast cell itself.¹ Freezing the tissue at -80°C ., preliminary to extraction, may render the tissue somewhat more brittle without, at this stage, affecting markedly the physical or chemical properties of the tissue components.²

The choice of solvent has considerable importance. So-called isotonic solutions of NaCl appear to be unsuited for the preparation of the tissue granules. In salt solutions, the granular material is highly unstable, has a tendency to aggregate and, on standing, deteriorates rapidly, leaving an insoluble residue. This effect is probably due to a great extent to the tendency of salt solutions to become acid, although neutralized salt solutions have also been found unsatisfactory. Equally good results have been obtained with the use of 0.005M Phosphate Buffer solution of pH 7.1 or a 0.0002N solution of NaOH.³ In the latter solvents, the tissue granules form homogeneous suspensions which have remained stable for many months, at 4°C . The use of phosphate buffer solutions, or slightly alkaline water, does not seem to influence the final chemical constitution of the material.

METHODS

Extraction. In preparing the extract proper, the disintegrated tissue mass was suspended in a volume of solvent, usually the alkaline water referred to above, corresponding to eight or ten times the weight of the original tissue. This suspension was centrifuged for 20 minutes at $2400 \times$ gravity,⁴ a step which removes the coarse tissue debris, cell fragments, free nuclei and, in general, tissue fragments roughly equal to, or larger than, particles of one or two μ in diameter. The supernate from this low speed centrifugation, referred to as the "tissue extract," is the preparation generally used for further fractionation in the high speed centrifuge. In order to lessen the effect of autolysis and "spontaneous" deterioration of the material, extraction and subsequent purification in the high speed

¹ Flint No. 0000, obtained from Charles B. Crystal Co., New York.

² Intact cells may remain viable upon freezing at -80°C ., and unaltered tissue granules can be obtained from them. In the isolated state, the particulate elements are very sensitive to the treatment, and a few minutes at -80°C is sufficient to cause loss of solubility and, apparently, denaturation of the proteins. This occurs even if a protein or normal serum has been added to the purified granules.

³ The concentration of these solutions is 0.7 and 0.008 mg. per cc. respectively.

⁴ International Equipment Co., Boston; Type S.B., Size 1, centrifuge in conjunction with conical head No. 283.

centrifuge were conducted in a refrigerated room, at 0° to 6° C. all accessories and solvents being chilled to 0° C. beforehand.

High speed centrifugation. The device used in our laboratory has been the multispeed attachment and N° 295 head, manufactured by the International Equipment Co., for their type S.B. Size 1, centrifuge. When using the full capacity of the cups provided in the centrifuge head, the maximum volume of liquid which can be centrifuged in one operation is 84 cc. This is obtained by using large lusteroid tubes of 14 cc. capacity.⁵ At top speed (approximately 18,000 r.p.m.), the centrifugal force developed in the middle of the tube, 4.4 cm. distant from the center of rotation, is about 18,000 times the force of gravity. Under these conditions, the haemocyanin of *Helix pomatia*, with a molecular weight of six millions and a diameter of about 24 μ , will be completely sedimented in the course of 4 hours (Claude, 1937). Particles 60 μ in diameter or larger would be completely separated by a centrifugation of one hour.⁶

In the separation of the tissue granules, the standard technique has been to use the centrifuge systematically at its full speed of about 18,000 r.p.m., varying only the time of centrifugation. In a typical experiment, the particulate components of the tissue extract were first spun down by a run of one hour in the high speed centrifuge. The sedimented material was then taken up in a small volume of water. Coarse particles were removed from this suspension by a short run of three to five minutes. The coarse sediment was resuspended in water, redeposited by a short run of three to five minutes, and discarded. The supernatant fluids from the different short runs were combined and saved for further purification in the centrifuge. The entire process, consisting in a long run of one hour, followed by two successive short runs of three to five minutes was repeated two or three times. During the long runs, the tissue granules become separated from the light elements of the extract while the short runs serve to remove the coarser particles.

If one assumes that particles of all sizes are originally present in the tissue extracts, it can be calculated that the above method will bring about the concentration of those particles ranging in size between 60 and 200 μ in diameter (approximate density, 1.3). The larger particles are discarded at a much greater rate than the smaller ones during the short centrifugal runs so that, in the end, the purified tissue fraction may be composed of a population of granules of various sizes, the largest being approximately three to four times the size of the smallest, with a predominance of particles with a diameter of 60 to 150 μ .

RESULTS

In the purified form, the material prepared from various sorts of tissues appears to be composed of small granules visible, under dark field illumination, as refractile bodies of nearly the same size, and showing Brownian motion. That the material is not homogeneous with regard to particle size is indicated by a rapid spreading of the sedimenting boundary when it is examined in the analytical centrifuge (Stern and Duran-Reynals, 1939; Claude and Rothen, unpub.) Whether the material represents a continuous series with respect to particle size or is composed of definite species of particles has not been ascertained, except in the case of normal liver where three components of different particle sizes and chemical properties have been separated.⁷ The data which follow refer especially to particles isolated from chicken tumor I tissue, by the above technique, and ranging in size between 50 and 200 μ . Physically and chemically these particles do not appear to differ from similar ones isolated from other animal tissues (Claude, 1940) and a description of their properties may be considered as typical for this kind of tissue granules in general.

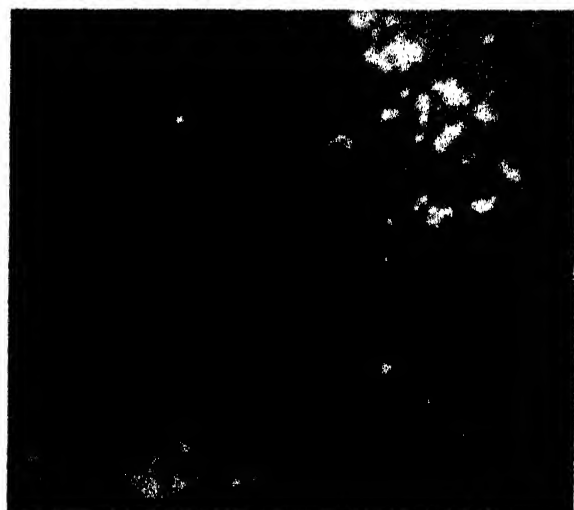
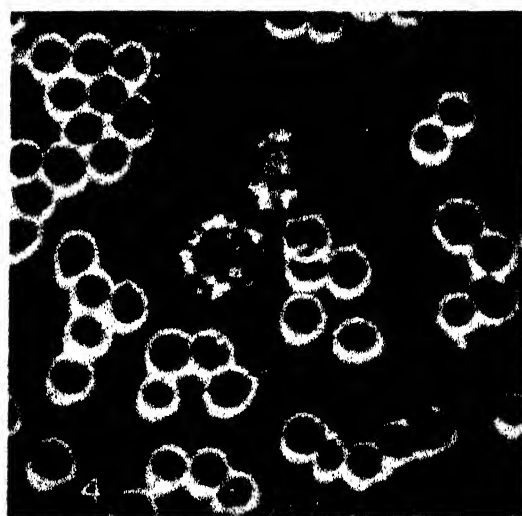
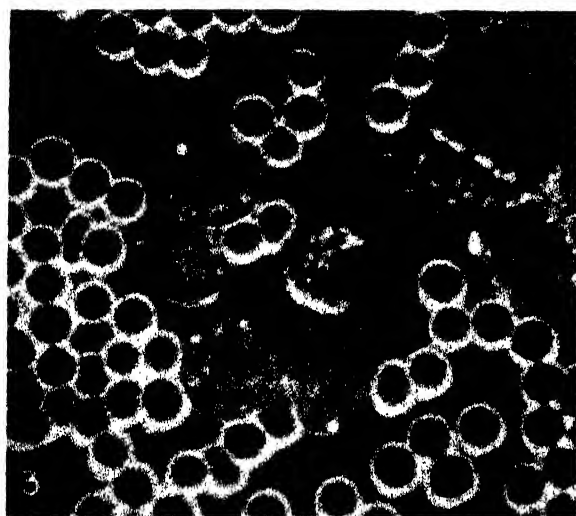
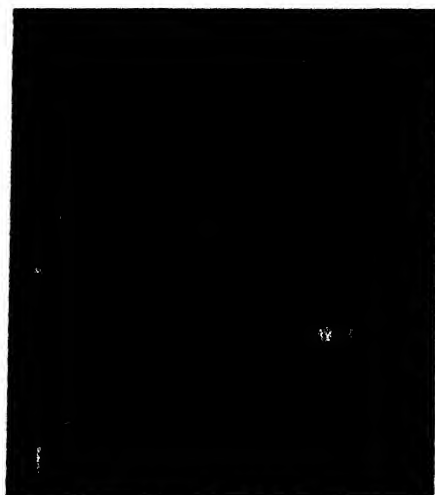
On chemical analysis, the particles are found to be composed essentially of two main portions, one lipid, the other largely protein in nature. The lipid portion accounts for 40 to 50 percent of the whole particle. About 75 percent of this lipid fraction is represented by phospholipids, as shown by phosphorus and nitrogen analysis. An interesting feature of this lipid fraction is that it gives a positive reaction with the fuchsin-sulfurous acid reagent of Schiff, indicating the presence of aldehyde groups in the material. The aldehyde components represent probably the substances isolated recently by Feulgen and Bersin under the name of acetalphosphatides (Feulgen and Bersin, 1939). These phospholipids are highly soluble in alcohol. In tissues, they are held responsible for the plasmal reaction of Feulgen and Voigt (1924).

The total nitrogen content of the complete particle is eight to nine percent. After removal of the lipids, the remaining protein portion shows a nitrogen content of 12.7 to 14.9 percent, and 1.23 percent phosphorus. The chief constituent of this protein portion appears to be a nucleoprotein of the ribose type. Color tests for pentoses are positive and there is indication of a maximum of absorption in the ultraviolet, in the region of λ 2600. From this protein material, a ribose nucleic acid has been isolated and was identified by phosphorus analysis, typical color tests and ultraviolet absorption spectrum. The pentose nucleic acid has been found to represent as much as 10 to 15 percent of the protein

⁵ Obtained from the Lusteroid Container Company, South Orange, New Jersey.

⁶ If the viscosity of the fluid is greater than that of water, the time of centrifugation should be increased proportionally.

⁷ Mitochondria can be observed directly in the living cell, under dark field illumination (figs. 2, 3, and 4). In this case also, mitochondria do not seem to be of absolute uniform size.



portion of the particle. A nucleic acid has been isolated from particles derived from chicken tumors, chick embryo, mouse embryo and mouse tumors, rabbit embryo and from the liver of guinea pigs by following a standard procedure (Claude, 1939). In each case, the nucleic acid appeared to be of the ribose type and tests for thymonucleic acid were negative.

Results obtained from analysis of particles of different origins emphasizes the fact of a uniform chemical composition, irrespective of the tissues from which the granules were derived. This indicates that particulate elements, having the general composition of a phospholipid-ribonucleoprotein complex, are present in normal and tumor cells. That this material may be an important constituent of the cell is shown by the fact that it represents at least as much as 3 to 12.4 percent, in terms of dry weights, of the entire cellular body.

RELATION BETWEEN TISSUE GRANULES AND MITOCHONDRIA

The occurrence, in tissue extracts, of a complex of definite chemical composition raises the question whether or not these elements may pre-exist in the form of similar bodies in the cytoplasm. The formed elements of the cell which, by their mass, represent an important part of the cellular body, are the nucleus, the Golgi apparatus and the chondriome. Since, in many instances, it can be shown that the intact nucleus is discarded through the preliminary centrifugation at low speed, it is apparent that the granules must derive from the cytoplasm. The substance of the Golgi apparatus has a relatively low density and moves towards the centripetal pole of the cell when the tissue is submitted to high speed centrifugation, as shown in the experiments of Beams and King (1934). On the other hand, a review of the general properties of mitochondria indicates that these elements possess many important features in common with the constituents of the purified material.

In some instances, it can be shown that the size of the purified particles falls in the range of sizes of mitochondria. This point can be illustrated by using a transmissible leukemia of the rat as the source of the granules. The leukemic element is a large cell of lymphoid type which normally involves the thymus, the lymph nodes and the circulating blood. Murphy and Sturm (1941) have shown recently that the disease could be propagated in the subcutaneous tissue where the malignant cells form large tumor masses, made up almost entirely of typical leukemic elements. Figure 1 shows a sus-

pension, in neutral water, of the purified granules separated from lymphoid tumors, as they appear under dark field illumination.⁸ Most of the granules are free, while a few, at the lower right corner of the figure, seem to be agglutinated. Photographs to illustrate the relative homogeneity of the suspension were difficult to obtain as the particles were in constant Brownian motion and only those in focus at the moment can be seen as little granules of nearly the same size. In size and shape, the free granules of Figure 1 can be compared with the intracellular granules which occur in the cytoplasm of living cells, as shown in Figures 2, 3, and 4. Whole blood was selected for dark-field examination of living cells because the cells can be observed in their own medium and thus subjected to the least possible injury. In the background of Figure 2, fibers of fibrin can be seen. In the case of Figures 3 and 4, heparin was added to prevent coagulation. Figures 1 to 4 are comparable in that they were made under the same conditions and at a magnification of 1000 \times . In the lower center of Figure 2, the cell containing large granules and a ring-shaped nucleus is a polymorphonuclear leucocyte, probably a neutrophile. The majority of the lymphoid cells which appear in Figures 2, 3 and 4 belong to the same leukemic strain from which the granules of Figure 1 were obtained. The intracellular, refractile, bodies of the lymphoid cells, which often appear to segregate around the nucleus, are mitochondria, as shown by simultaneous examination of the same or similar cells in the ordinary microscope, in conjunction with Janus Green. In Figure 4, free, purified, granules were added to the plasma and are seen beside the cells. From a study of the above figures, it can be concluded that the granules isolated from leukemic tissue are similar in size and shape to the mitochondrial elements of the leukemic cell. Although the evidence does not provide a direct proof that they are identical, it supports the view that the purified granules are free mitochondria.

The assumption that the granular elements under study may represent mitochondria is also supported by the chemical composition of the material. Since the work of Regaud (1908) the idea that mitochondria are made up of phospholipids and proteins has been accepted by many workers (Cowdry, 1918; Guilliermond, 1934). The occurrence together of nucleoproteins and of phospholipids, highly soluble in alcohol and presenting the properties of aldehydes, would explain the response of mitochondria to histological dyes and fixatives. The

* Cardioid Siedentopf Condenser (Zeiss); carbon-arc lamp; magn. 1000 \times diameter.

Figures 1 to 6. Dark-field, magnifications \times 1000. Rat leukemia. "Small particles," purified, in neutral water. Dark-field. Figure 2. Rat leukemia. Whole blood showing cytoplasmic granules in leukemic and normal cells. Figure 3. Rat leukemia, heparinated blood. Cytoplasmic granules in lymphoid cells. Figure 4. Rat leukemia, heparinated blood. Cytoplasmic granules in lymphoid cells. Purified particles added to plasma. Figure 5. Guinea pig liver. "Small particles" agglutinated at pH 6.0. Phosphate buffer. Figure 6. Guinea pig liver. Purified "Bensley" granules in neutral water.

presence of large quantities of nucleic acid could account for the affinity of mitochondria for basic dyes under certain conditions.

Mitochondria are known to be destroyed by many ordinary fixatives, especially by those containing acids (Cowdry, 1918; Guilliermond, 1934). They are sensitive to heat and seem to disintegrate when cells are brought to 48°-50° C. (Policard, 1912; Cowdry, 1917). In this respect, it may be of interest to study the effect which heat and acids have on the isolated granules.

By testing the solubility of the purified particles in buffer solution of different pH's, it is found that immediate aggregation of the particles takes place as soon as the medium becomes slightly acid, this being already evident, at pH 6.0, by a marked increase in the opalescence of the suspension. Figure 5 shows microscopic clusters of the particles, a few minutes after the suspension had been brought to pH 6.0. Below that point, opalescence increases rapidly, with massive flocculation between pH 4.5 and 2.5. A point of minimum solubility can be found near pH 3.5. In collaboration with Dr. A. Rothen (Claude and Rothen, 1940), it has been shown that the purified material disintegrates rapidly in acid solutions, with the liberation of a constituent of lighter molecular weight which is no longer sedimentable by prolonged centrifugation at 18,000 \times gravity. It is assumed that, under the effect of acid, namely at or below pH 3.5 the phospholipid-ribonucleoprotein complex becomes disrupted while the nucleoprotein, or nucleic acid, is set free into solution. This is supported by the fact that the component of low molecular weight which diffuses in acid solutions presents an ultraviolet absorption curve characteristic of nucleic acid and gives positive tests for pentoses.

Similarly, heating the purified material at 50° C. for 30 minutes is sufficient to liberate from the particles a constituent of low molecular weight which, from its absorption spectrum in the ultraviolet and characteristic color tests, appears to be nucleic acid (Claude and Rothen, 1940). Mere freezing at -80° C., or drying, of the material may have the same effect.

From these observations we may infer that the cytoplasmic granules, presumably mitochondria, may react within the medium of the cell in the manner of the free granules. Under physical or chemical injury, the nucleic acid, or nucleoprotein molecule, may lose its connection with the rest of the complex, and diffuse in the surrounding medium, where it may account for the so-called basophilia of the cytoplasm. This would be in agreement with the fact, recognized long ago by cytologists, that the cytoplasm of actively growing cells or of cells with an active metabolism is generally highly basophilic and, in the same time, rich in mitochondria.

Caspersson and Schultz (1940) suggested recently that ribose nucleic acid compounds present in the cytoplasm may have their origin in the synthetic

activity of the nucleus, the synthesis of ribonucleic acid occurring at or near the nuclear membrane. This view was principally based on the observation that ultraviolet absorbing substances may occasionally be found, accumulated around the nuclear membrane. Mitochondria are often seen arranged near and around the nucleus, as illustrated in Figures 3 and 4, and the release of nucleic acid around the nuclear membrane, under the influence of fixatives, might account for greater absorbing power of this area for the wave length region of λ 2600. It must be pointed out that Caspersson (1940) and Caspersson and Schultz (1940), used fixatives containing large quantities of acid. This treatment may be expected to destroy mitochondria completely. (Cowdry, 1918; Guilliermond, 1934), allowing light-absorbing elements to diffuse into the surrounding cytoplasm. Under such conditions, occurrence of free ribonucleic acid in certain areas of the cell may be an artifact resulting from the destruction of mitochondria in that region. From these considerations, it seems that the problem of the origin of ribonucleic acids in the cytoplasm may have to remain open until more is known about the mode of generation of mitochondria themselves.

CYTOPLASMIC GRANULES OF THE GUINEA PIG LIVER

R. R. Bensley has been a pioneer in the separation of particulate components of normal cells. Bensley and Hoerr in 1934, and Bensley in 1937, reported on the isolation of granules from the guinea pig liver and referred to these granules as mitochondria. Since the Bensley granules were considerably larger than the particles isolated in our laboratory, and since they appeared to have a somewhat different chemical composition, a comparative study of the two kinds of granules became necessary. The results of a systematic study of the particulate components from the cytoplasm of guinea pig liver cells are recorded below.

According to Noël (1923), who made an extensive study of the mouse liver, mitochondria appear as discrete filaments or small granules in the cytoplasm, together with much larger granules which may be as much as two to three μ in diameter. Noël offered evidence to show that the large secretory granules result from a progressive transformation of mitochondria. The large granules, which he terms *plasts*, would be homologous with the chloroplasts of plants.

As mentioned above, the cell membrane of the hepatic cell breaks up under slight injury. When fresh liver tissue, crushed by simply passing through a meat grinder and suspending in saline or water, is examined under high power or in a dark field microscope, it is found that the majority of the cells have disintegrated, releasing the cytoplasmic granules in the medium. The so-called secretory granules (Noël, 1923; Kater, 1931, 1937; Bowen, 1929) appear as small spheres of considerable size

which may vary, approximately, from 0.5 to 3 μ in diameter. These secretory granules in many respects resemble fat globules and, under transmitted light or in dark field illumination, appear as perfectly spherical objects with a brilliant surface, outlining a dark center (fig. 6). On the other hand, the smaller elements of the cell, which are homologous with the small particles isolated previously from other tissues, are not seen clearly except under dark-field illumination, and then appear as dense, refringent bodies, generally granular, but sometimes elongated, as indicated by a tumbling motion in the fluid. From mere inspection, and by comparison with the secretory granules, it is apparent that the size of these small particles is less than 0.5 μ in diameter.

In our experiments, the large and small granules of the guinea pig liver were separated by the following procedure, the alkaline water being used throughout for extraction and the washing of the different fractions.

The liver tissue was passed through a meat grinder, then forced through a screen of one mm. mesh, and the resulting pulp suspended in eight times its weight of water. The suspension was immediately centrifuged for one minute at 2000 r.p.m. in a horizontal centrifuge. This step was found to remove most of the liver fragments, the cells which had remained intact, the free nuclei and the red corpuscles. The supernatant fluid, or extract proper, which contained practically all the organic components equal to, or smaller than, three μ diameter, was spun at 18,000 r.p.m., in the high speed centrifuge, for exactly five minutes. At that speed, a five minute run was sufficient to bring down practically all the large secretory granules. The sediment was saved for further purification in the centrifuge. The small particles which had remained in the supernate, were sedimented by a long run

purified in the high speed centrifuge, the "long run" in this case being five minutes centrifugation at 18,000 \times gravity. The procedure consisted in suspending the material in water and sedimenting it again at high speed, four times in succession. It was found that the usual "short runs" were not necessary, because the coarse elements contaminating the granules became firmly packed at the bottom of the tube at each centrifugation and could be left behind, while the secretory granules had no tendency to aggregate and were readily resuspended in the slightly alkaline water.

By the above technique, two definite fractions were obtained from the guinea pig liver: a) the usual tissue particles, ranging in size between 60 and 200 m μ , and considered to be mitochondria or fragments of mitochondria; these particles from liver are homologous with those shown in Figure 1; and b) the secretory, or Bensley granules, ranging in size between 0.5 and 3 μ . A purified suspension of the Bensley granules are shown in Figure 6. Only the granules which happen to be in focus appear with their true dimensions. These are the globular elements showing a refringent outline and a dark center. A comparison with Figure 1 may give an idea of the difference in size and shape between the two kinds of cytoplasmic components.

The amount of small particles obtained from the guinea pig liver was about 7.7 percent, in terms of dry weight, of the whole organ, a value which is in agreement with the yield obtained previously from other tissues (Claude, 1940). On the other hand, the Bensley granules were found to represent only 2.5 percent of the cellular body.⁹ These last results were unexpected since the large granules are conspicuous elements and, in the living state, appear to fill up the entire cytoplasm.

Chemical analysis of the two fractions is summarized in Table 1. The figures obtained for the

TABLE 1. GRANULES FROM GUINEA PIG LIVER. CHEMICAL COMPOSITION OF TWO FRACTIONS ISOLATED BY DIFFERENTIAL CENTRIFUGATION.

Exp. No.	Guinea Pig	Fraction	N	P	C	H	Ash	S	Cu	Lipoids (total)	Amount purified fraction in liver
1	Normally fed	Large granules	percent 11.12	percent 0.82	percent 53.31	percent 7.93	percent 2.74	percent —	percent 0.020	percent 22.4	percent 2.4
2	Fasted 72 hours	Small particles	9.47	1.15	55.25	8.43	4.97	—	—	40.8	8.9
		Large granules	12.24	0.91	53.51	8.07	3.56	—	0.034	24.0	3.0
3	Normally fed	Small particles	8.72	1.23	56.40	8.63	4.06	0.87	0.013	—	5.5
		Large granules	12.17	0.93	53.17	7.78	4.40	1.16	0.030	—	2.1

of one hour at the same speed. The small particles were purified in the usual manner, by a series of centrifugation at high speed for alternate runs of one hour and of three minutes, respectively.

The secretory granules which formed a loose sediment, were readily resuspended in water. They were

small particles agree closely with the values obtained previously for other tissues (Claude, 1940) although the phosphorus content of the liver particles appears to be definitely lower. The large secre-

⁹ These values are probably too low, small quantities of both fractions being lost during the process of purification.

tory granules possess a chemical make-up of their own. The lipoid content of the large granules was found to be no more than 22 to 24 percent against the usual 40 to 50 percent for the small particles. The large granules are also characterized by a much higher nitrogen content and less total phosphorus. Both the secretory granules and the small particles were found to contain an appreciable amount of copper.

It has been claimed that feeding may have a considerable influence on the size and the abundance of the large liver granules (Noël, 1923; Kater, 1931, 1937). Homologous fractions, obtained from the liver of guinea pigs which had fasted for 72 hours, showed no significant differences in amount and no differences in chemical composition when compared with the fractions derived from animals which had been normally fed (see table 1). This indicates a considerable stability in the make-up of the large granules and suggests that they are not merely centers of condensation of metabolites, as frequently suggested, nor are they the locus of segregation of different products of secretion, which would be subject to variation, according to the diet of the animal or the activity of the cell.

Since ribonucleoproteins have been found to be constant constituents of the small tissue particles, an attempt was made to separate nucleic acid from the liver granules. 42 mg. dry substance of both the small particles and the Bensley granules were treated according to the technique used in previous work (Claude, 1939). The "nucleic acid" solutions

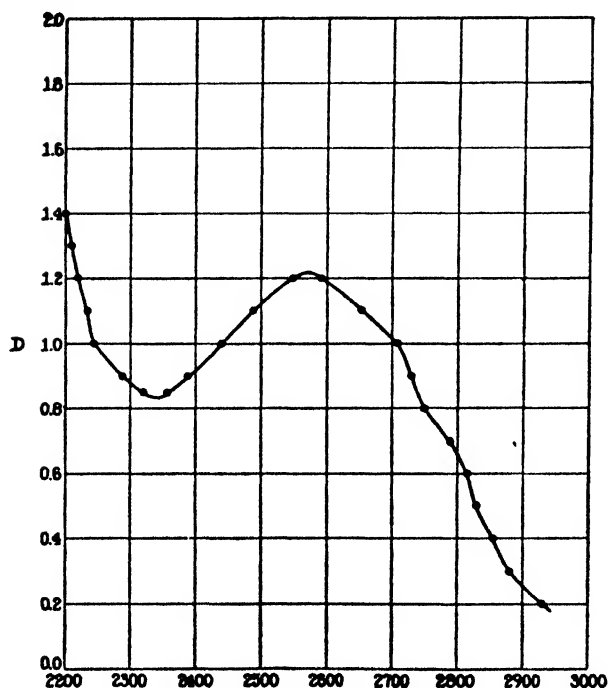


Figure 7. Ultraviolet absorption curve of nucleic acid prepared from the "small particles."

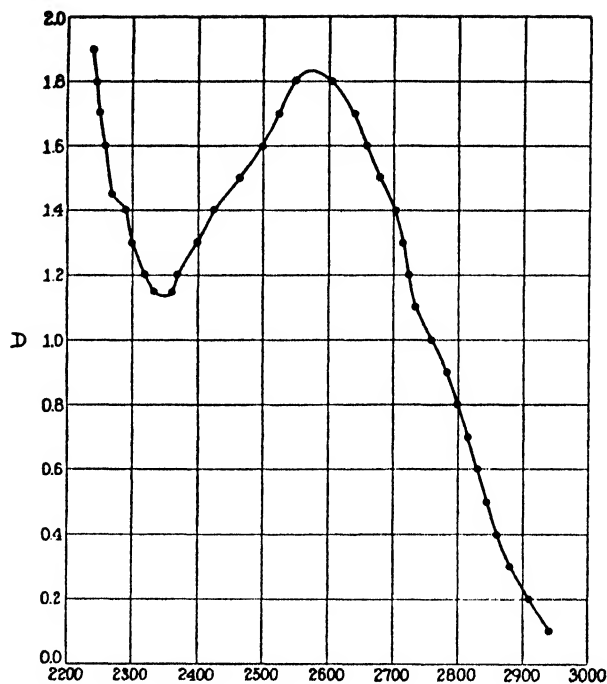


Figure 8. Ultraviolet absorption curve of nucleic acid prepared from the "large granules."

from both sources gave a negative biuret test, positive tests for pentoses and an absorption spectrum in the ultraviolet, typical of nucleic acid. As on previous occasions, the maximum was found at λ 2575 (12, 20). Measurements of absorption in the ultraviolet were made by Dr. G. I. Lavin. Figures 7 and 8 represent the absorbing power exhibited by the "nucleic acid" solutions derived from the small particles and the Bensley granules, respectively. During the separation of nucleic acid, the volume of the solutions was kept constant except that, before absorption measurements, the solution derived from the small particles was diluted with an equal volume of water. The absorbing power of the latter fraction, therefore, appears to be slightly greater than that of the similar fraction prepared from the large granules.

In acid or alkali, the Bensley granules behave very much like the small particles. At pH 6.0, the granules were already strongly agglutinated, and were completely precipitated between pH 2.5 and 4.5. As in the case of the small particles, a point of minimum solubility was also found near pH 3.5. Between pH 7.0 and 10.0, the granules remained free in the solution, and suspensions were relatively stable. Due to their large size, the large granules may sediment on standing, in contrast to the suspensions of small particles which remain homogeneous for many months. Above pH 10.0 there is a decrease in the opalescence of the suspension.

These similarities, and other properties which the small particles and the large granules have in com-

mon, especially the presence of ribose nucleic acids, suggests a possible common origin for these cytoplasmic elements. The findings seem to support the opinion of Noël (1923) who has offered evidence to show that the large granules, which he named *plastids*, have their origin in a progressive transformation of the smaller elements, or mitochondria. According to this view, the large granules would be, in some respects, specialized mitochondria. Whether a chemical continuity can be found between mitochondria and the large granules must be the subject of further work.

FURTHER FRACTIONATION OF LIVER EXTRACTS

In the preceding experiments, the liver extract was submitted to high speed centrifugation for periods not exceeding one hour.

In the next experiment, the long run at 18,000 \times gravity was continued for two consecutive hours and the sediment fractionated and purified further by differential centrifugation. Altogether, three different kinds of granules, readily distinguishable from their physical characteristics, were separated from extracts of guinea pig liver. a) One fraction is brought down by five minutes centrifugation. This fraction is composed of large granules ranging in size between 0.5 and 3 μ in diameter, and corresponds to the "secretory" granules described above. Either in dilute or in concentrated form, the material presents itself with a yellow color, as already noted by Bensley (personal communication). b) A second fraction is completely sedimented by 45 to 60 minutes centrifugation at high speed. This second fraction corresponds more or less to the "small particles" referred to above, and may contain elements varying in size between 60 and 200 $m\mu$ in diameter. In the centrifuge, this fraction forms jelly-like pellets which are perfectly transparent and colorless. Suspensions of this material are milky-white and strongly opalescent. c) A third fraction, separated by two hours centrifugation at high speed, is made up of smaller particles which, from their rate of sedimentation, may be estimated to vary in size between 40 and 60 $m\mu$ in diameter. In the centrifuge, the latter fraction appears as a jelly-like material, completely transparent and cherry-red in color.¹⁰ Water suspensions of this fraction are pink in color and opalescent. When the two small components are centrifuged together the first to come down are the unpigmented particles, the red particles sedimenting at a much slower rate. The two components remain separated by a sharp boundary, indicating that an appreciable difference exists between the sedimentation rates of the two

¹⁰ The possibility that this component may come from the blood has not been ruled out. The livers for the above experiments were obtained from animals which had been killed by a blow, then allowed to bleed profusely. The fact that this fraction represents a large proportion of the liver, i.e., 6.8 percent of the whole organ, makes it unlikely that the red particles may derive from the circulating blood.

species of particles. In terms of dry weights, the amount of material separated from liver tissue was 3.0 percent for the Bensley granules, 4.6 percent for the colorless component and 6.8 percent for the red particles. Together, these elements represent 14.4 percent of the whole organ.

OCCURRENCE OF COPPER IN THE CYTOPLASMIC GRANULES OF VARIOUS CELLS

Yeast cells contain granules of various sizes which, by appropriate means, may be extracted from the cell. However, the procedure adopted for their preparation may affect markedly the final chemical composition of the purified granules. When fresh cells (Brewer's yeast) were extracted in the cold, by purely mechanical means, the purified product was a transparent, jelly-like material which was red-purple in color.¹¹ Physically and chemically, the yeast granules isolated in this manner appear to be homologous with the granules obtained previously from other cells. On the other hand, a different material was obtained when extracts of dried yeast were incubated 3 hours at 40° C. prior to fractionation in the centrifuge. The peculiarity of the purified pellet was that it presented a blue color, in some respects resembling the color of haemocyanins. Results of elementary analysis of this fraction are given in Table 2. Under ultraviolet light, the puri-

TABLE 2. FRACTIONATION OF BREWER'S YEAST BY DIFFERENTIAL CENTRIFUGATION. CHEMICAL COMPOSITION OF PIGMENTED GRANULES.

	Percent
N	11.73
P	0.30
C	53.15
H	7.80
Ash	1.65
Cu	0.116

fied substance did not show appreciable fluorescence. On chemical analysis, the material was found to contain 0.116 percent of copper. This value does not seem to be negligible if we consider that the copper content of the respiratory pigment of *Limulus* is 0.173 percent (Redfield, 1934).

Similar fractions from other sources were found to contain copper also, but in lesser proportions. The small particles isolated from the malignant cells of a mouse leukemia (MacDowell, 1939-1940) were found to contain 0.023 percent copper. The copper content of the small particles from guinea pig liver was 0.013 percent, against 0.034 percent for the large "secretory" granules (table 1). The latter figure represents an appreciable amount of the metal since it corresponds to 20 percent of the copper content of *Limulus* haemocyanin. There is

¹¹ Work undertaken in collaboration with Dr. Dean Burk and Dr. Charles Winzler.

evidence that the copper present in the cytoplasmic granules is associated with a protein. The possibility is being investigated that the copper compound may play the role of a respiratory pigment in the cell.

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DISCUSSION

COLE: The maximum in the absorption spectrum of thymonucleic acid is found by us to be at λ 2575. I would not like to say why it shifts before more is known about it.

CLAUDE: The maximum of absorption shown by our purified particles or by the "ribonucleic acid" fraction derived from it is generally found in the neighborhood of λ 2575. However, solutions of highly purified thymonucleic acid, or purine and pyrimidine bases, mixed in the proportion found in nucleic acids, gave us a maximum of absorption at λ 2600.

COLE: Would you tell in more detail how you extracted your sample from the centrifuge tubes? Do you reach it with a pipette?

CLAUDE: The usual procedure is to add a few drops of neutral water, rub up the pellet with a glass rod, add more water and finally, pump the material

up and down in a capillary pipette until a homogeneous suspension is obtained.

SCHULTZ: The distribution of nucleic acid in the formed bodies of the cytoplasm is an interesting point. Caspersson and I have considered the possibility that nucleic acid might be in the form of mitochondria and Golgi, but in some of our material, it seems unlikely that such is the case. The question is whether mitochondria are autonomous or whether they have a dependence upon the nucleus. They do perform regular manoeuvres in some cases during mitosis. One hypothesis is that ribonucleic acid synthesis in connection with the nucleus might give rise to complexes such as Claude describes. It should be noted that another system, the ergastoplasm, does not seem to have any of the ribonucleic acid compounds associated directly with the nucleus. For example, in the pancreas, the ergastoplasm is in the form of long fibers, with a high concentration of ribonucleic acid, and the relation with the nucleus is not clear.

Are the copper proteins of the granules related to tyrosinase?

CLAUDE: The evidence for the moment is that ribonucleic acid in the cytoplasm occurs in the form of small granules, some of these being undoubtedly mitochondria. Whether mitochondria can synthesize ribonucleic acid or derive it from some other system is a problem which involves the mode of formation of mitochondria themselves. At the moment, there is no information which would permit one to connect directly the production or the growth of mitochondria with the activity of the nucleus.

In recent years, the majority of workers have come to consider the ergastoplasm as an artefact produced, as a rule by fixatives containing strong acids. It is rare to find well preserved mitochondria and ergastoplasm simultaneously in the same preparation and the suggestion has been made that the ergastoplasm was merely poorly fixed mitochondria. A review of the early work on the ergastoplasm may be found in a paper of Bowen (1929).

We have had no occasion yet to investigate the possible relation between the copper proteins of the granules and tyrosinase.

SCHULTZ: The recent discussion of Ries does not agree; he has worked with the secretory cycle in the mouse pancreas and has followed the mitochondria in the living cell with Janus green. He attributes separate activity to the ergastoplasm.

I do not mean to imply that I think mitochondria are formed in the nucleus. The problem as I see it here is how the nucleus regulates the action in the cytoplasm.

GATES: Aggregations of mitochondria around the nucleus in prophase are often mistaken for spindle fibers.

WRIGHT: In rodents, the pigment granules make their first appearance in the mitochondria, but their characteristics are wholly determined as far as is

known by genes. This seems to indicate a close relation to the nucleus. At least three of the loci in the guinea pig affect the nature of dopa oxidase, and thus may be related to the copper content of the mitochondria.

PAINTER: During growth of the germinal vesicle of the toad, there is manufactured in the nucleus an enormous number of nucleoli which contain Feulgen-positive granules found by Brachet to be ribonucleic acid. The nuclear wall undergoes a series of movements and the granules disappear. He never finds any granules in the cytoplasm. This may be accidental but it is obvious that ribonucleic acid is formed in the nucleus.

CLAUDE: Ribonucleic acid does not give the Feulgen reaction. The origin of mitochondria is an interesting problem. There is no good evidence that mitochondria are able to multiply by division except perhaps in the interesting case of the scorpions, studied by Wilson and Pollister. In this connection we must remember that many of the mitochondria which we can isolate by centrifugation are too small to be seen in the ordinary microscope. Under these conditions, it is difficult to judge whether they can increase by division or are formed *de novo* in the cytoplasm.

COHEN: Have you any evidence for acetalphosphatides in the Feulgen reaction? Purines give the reaction. How can you be sure that purines are not present in the granules? Bensley has data on this.

CLAUDE: The purified phospholipids give a positive test with the reagent of Schiff. The nucleic acid present in the granules is of the ribose type and does not give the reaction. From information received from Dr. Bensley, it seems that the lipids of the fraction isolated by him react also with the reagent of Feulgen.

JAKUS: Which phospholipids are involved?

CLAUDE: We have no detailed information regarding the kind of phosphatides present in the lipid fraction except that acetalphosphatides are probably present.

GREENSTEIN: Are your analytical data repeatable for different preparations?

CLAUDE: Duplicate experiments give very close values, as shown in the first table, where material obtained from normal and fasted guinea pigs gave almost identical results.

GREENSTEIN: The rate of spontaneous denaturation of tissue proteins is extremely rapid.

CLAUDE: The preparation of our material is always carried out in the cold, between 0° and 10° C. Denaturation of the proteins is immediately noticeable by the fact that the granules are no longer soluble. The material clumps together and nucleic acid is lost.

COHEN: In examining the heavy fraction of lung, I have been able to separate nucleoproteins from phospholipids completely. So it seems important where, in the gross microscopic pieces, these are actually associated.

CLAUDE: Fractionation of the tissue is performed in the cold and by purely mechanical means. There is little doubt, in my opinion, that the granules exist in the cell as complexes of ribonucleoproteins and phospholipids.

GREENSTEIN: The thymonucleoprotein fraction of liver also has phospholipids associated with it, but there are two types of lipids, one readily removed with ether, the other requiring hot alcohol and acetone.

ANSON: There seem to be two kinds of heavy particles in specialized cells. In enzyme study, it is valuable to concentrate on specialized cells such as the stomach cells, since some properties are more readily accessible in the particles of such cells.

What have you found are the effects of freezing and thawing on the heavy material?

CLAUDE: Intact cells can be frozen without affecting to any appreciable degree the properties of the granules, which may be extracted subsequently by the usual technique. In the purified form, however, the tissue particles are immediately destroyed by freezing, as shown by loss of solubility and separation of nucleic acid.

SPECIFIC PROPERTIES OF FILMS OF PROTEINS

ALEXANDRE ROTHEN, BACON F. CHOW, R. O. GREEP, AND H. B. VAN DYKE

The aim of this article is to present certain facts dealing with specific reactions of films of certain proteins important in biology.

The amount of experimental work accumulated in the last few years on surface films of proteins and related substances is quite considerable. However, the literature on the subject of specific reactivity of protein films offers contradictory results. Some confusion has arisen from the indiscriminate use of the term monomolecular layer or monolayer. When dealing with small organic molecules the term monomolecular film is not ambiguous. These molecules have a rather rigid structure and the fact that they are located at an interface solid-gas or liquid-gas, merely orients them in a certain direction. In the case of proteins, however, the term monolayer is ambiguous and needs to be sharply defined for the following reasons: From data, which have been obtained mainly with the help of the ultracentrifuge, we know that proteins have definite molecular weights in a range varying from 13,000 (ribonuclease) (Rothen, 1940-1941) to 900,000 (antipneumococcus horse globulin) (Svedberg and Pedersen, 1940). We also have a good idea of their shape which can be approximated by ellipsoids with axis ratios of the order of 2/1 (ribonuclease) to 30/1 (zein). From these figures one can estimate the thickness of a monolayer of such molecules, assuming no orientation or orientation along one of the axes. Thicknesses of the order of 25 to 100 Å are obtained in this way. However, it is an experimental fact that when a drop of a protein solution is placed on the surface of a buffer solution at a pH near the isoelectric point of the protein, under favorable conditions the protein spreads on the surface and the film thus formed has a thickness of only 6 to 10 Å. Most proteins, whatever their molecular weight, form films of about this thickness, which corresponds to the average thickness of an extended polypeptide chain. The globular shape of the protein has disappeared during the spreading process; the molecule has been unfolded and can be called denatured. On the other hand, if protein molecules are directly adsorbed on a solid surface it is found that the thickness of the layer is at least that corresponding to the diameter of the molecule calculated from the molecular weight, but very often layers very much thicker are obtained when the solution is near the isoelectric point (Clowes, 1939). Between the completely unfolded state and the completely native state intermediate steps may occur. The fact that a film is obtained by spreading does not necessarily mean that the molecules are unfolded. It might be expected that thick films of folded or native molecules would retain the activity exhibited by the molecules

in solution. One must therefore differentiate between the two types of films when speaking of a monolayer of protein.

Experiments on the reactivity of films of enzymes have been carried out by a number of workers. Four enzymes have been studied, namely, pepsin, urease, catalase, and saccharase. Gorter (1937) and Langmuir and Schaefer (1939) obtained pepsin in an unfolded state. They reported that the activity was not lost. Inconclusive results were obtained with catalase (Langmuir and Schaefer, 1938; Harkins, Fourt and Fourt, 1940). Urease seemed to lose its activity (Langmuir and Schaefer, 1938). Recent experiments by Sobotka and Bloch (1941) on saccharase showed that "Built-up saccharase films of a thickness up to 45 Å retain the full enzymatic activity." Unfortunately the very thick layers obtained by these authors indicate that the saccharase molecules were not unfolded, and were probably in their native state.

All the new experimental data which are presented in this article are concerned with films of unfolded protein molecules. Insulin and two hormones of the pituitary glands, as well as the antiserum of one of the pituitary hormones, were studied in this investigation.

EXPERIMENTAL TECHNIQUE

The films were formed in the following way. A few drops of a one percent solution of the protein to be spread were placed on a piece of filter paper which had been shown to be free from spreadable material. The paper was slowly brought into contact with the clean surface of the trough. Two troughs were used, a small one 50 × 14 cm. made of plastic material (plexiglas) and a large one 90 × 30 cm. made of enameled brass. Both trays had a well at one end for the transfer of the film to the chromium slides, according to the technique of Blodgett and Langmuir (1937). Films were compressed mechanically by chrome plated brass barriers. Piston oil, as described by Langmuir and Schaefer (1937), was never used since it was found that contamination was unavoidable, especially when the films stayed for a long time on the surface. The small trough was enclosed in a dust-proof box to avoid contamination through the air. Film pressure was measured by the Wilhelmy balance method as recently described by Harkins and Anderson (1937). The sensitivity was of the order of one dyne per centimeter of the scale which could be read within a millimeter. The thickness of the film was determined optically after transfer to chrome plated slides according to the method of Blodgett and Langmuir (1937). The slides had a width of 15 mm. and each of the 10 steps of the optical gauge was 1 mm. wide. The

values for the thickness of the films given in the tables are only relative since the refractive index of the films used in the calculation was not determined but was assumed to be the same as that of the stearic-stearate film, *i.e.* $n_D = 1.495$. The pressure applied to the film for the transfer varied from 2 to 10 dynes. A pressure of two dynes was sufficient for the transfer on the "down trip" but at least seven dynes were necessary on the "up trip" to prevent the layers from slipping back into the trough. Plates coming out of the tray were wet, as is normally the case with protein films. If the plates were washed before drying, only one layer stayed on. If they were dried first, both layers stayed on but in a few instances the top layer could be washed away. No significant difference could be observed in our results whether a double layer or a single layer was used. In the experiments reported here either single layers or double layers were used.

Metakentrin, a gonadotropic hormone of the anterior lobe of the pituitary gland (Shedlovsky, Rothen, Greep, van Dyke and Chow, 1940), the oxytocic-pressor hormone of the posterior lobe recently isolated in pure form (van Dyke, Chow, Greep and Rothen, 1941), and crystalline insulin¹ seemed ideal proteins for a study of the effect of unfolding on biological activity, and, in the case of metakentrin, on serological phenomena. These substances were particularly suitable on account of their purity and also because of the extreme sensitivity of the biological test, the limit of the test being 1 γ protein nitrogen in the case of metakentrin (anterior prostate of immature hypophysectomized male rat) and, for the posterior-lobe hormone, 0.05 γ N (oxytocic effect assayed by action on the fowl's blood pressure or on the isolated guinea pig uterus) or 0.01 γ N (pressor effect estimated by diuresis-inhibition in the rat). For insulin, the threshold of the test was 0.06 γ protein N (convulsions in the starved albino mouse).

These substances were spread on the surface of the trough, buffer solutions near the isoelectric point being used (phosphate or veronal buffer pH 7.4 for metakentrin, acetate buffer pH 4.6 for posterior lobe hormone, and acetate buffer pH 5.6 for insulin). Metakentrin with a molecular weight of 90,000, the posterior lobe hormone with a molecular weight of 30,000, and insulin with a molecular weight of 41,000 gave completely unfolded films of 7 to 9 Å thick. The films were unquestionably extended.

The technique of the serological experiments is described later.

BIOLOGICAL ACTIVITY

Films of metakentrin, of the posterior lobe hormone, and of insulin, all 7 to 9 Å thick, were spread on the large tray. They were mechanically compressed to about 8 dynes, and were collected from

the surface of the tray with a ring of platinum wire, the surface enclosed by the ring being about 8 cm.² The collection of the film was undertaken a few minutes after the completion of spreading which occurred very rapidly. The amount of spread protein collected in that way for each experiment varied from 300 γ to 1100 γ , a sufficient amount for nitrogen determination and biological test. The amount of protein film could also be estimated from the area collected, the thickness of the film, and the density of the protein which was assumed to be 1.3. The agreement between the figures obtained in this way and those obtained from nitrogen determination is shown in Table 1.

TABLE 1

Hormones	Estimated from area	Found by nitrogen determination (micro-Kjeldahl*)
	γ protein	γ protein
Metakentrin	450	350
Metakentrin	500	600
Metakentrin	1200	1380
Posterior lobe	750	880
Insulin	700	708
Insulin	780	1110

* Accuracy ± 5 percent.

Assays of metakentrin showed conclusively that much, if not all, of the activity (> 90 percent) had disappeared as a result of spreading this protein. The biological results are given in Table 2. It is

TABLE 2. BIOLOGICAL ACTIVITY OF METAKENTRIN IN HYPOPHYSECTOMIZED IMMATURE MALE RATS BEFORE AND AFTER SPREADING

Experiment	Dosage γ nitrogen injected	Number of rats	Average weight in mg. of anterior prostate		
			Film	Native hormone	Control
A	—	5			7.92
	2	6		12.8	
	2	6	7.10		
	10	3	6.77		
B	—	8			6.03
	2	8		11.9	
	20	4		24.2	
	20	2	10.6		
C	—	11			6.7
	2	5		11.1	
	5	6		14.1	
	5	5	6.3		
	26	5	8.9		

clear that the ability of the hormone to stimulate the interstitial cells of the testes so that secreted androgen causes prostatic hypertrophy is nearly abolished after metakentrin has been spread as a protein film. It is regrettable that larger amounts

¹ Activity of sample: 24.2 units per mg.

of spread hormone were not available so that the loss of potency could be more exactly estimated.

The data of Table 3 indicate that 97 to 99 per cent of the activity of the posterior lobe hormone was destroyed by spreading the protein as a film. However, it must be remembered that the oxytocic and pressor activities have been nearly completely separated by Kamm and his co-workers and represent, we believe, the active fragments of the protein which we used. Therefore it was possible that these fragments were to be found in the underlying fluid. If this were the case, the activity per unit nitrogen of protein in the fluid should be increased appreciably since the activity of this fluid would depend upon native protein inevitably dissolved in

Unfolding of the native insulin molecule has no effect on the biological activity. This fact strongly favors the view that one or more small structural units within the molecule, rather than the whole molecule, are responsible for the biological action of insulin. It could be argued, of course, that the spreading process is a reversible one in this case and that at the time the film is collected from the surface and suspended in the original solution, the native or nearly native configuration is regained. This possibility is very unlikely. The collected film at pH 5.6 gave an opalescent solution showing that some aggregation had occurred. When this solution was acidified the opalescence disappeared. Activity of the same order was observed, using the acidified

TABLE 3. BIOLOGICAL ACTIVITY OF ACTIVE PROTEIN OF THE POSTERIOR LOBE OF THE PITUITARY BODY BEFORE AND AFTER SPREADING

Experiment	Material assayed	γ N per U. S. P. unit*		
		Oxytocic activity		Pressor activity
		G.P. uterus	Fowl B.P.	Diuresis-inhib. in rat
A	Film	>143	>330	1000
B	Film		500, 330	500
	Hormone in buffer diluted, then concentrated...		10	10
	Hormone in buffer subjacent to film, later concentrated		32, 12	36

* 1 U.S.P. unit \equiv 10 γ protein nitrogen of pure native hormone.

spreading the film (one fourth of the total protein used) together with the film's active fragments. Therefore, in experiment B, the activity of the fluid as well as that of a similar dilute control solution were determined. The underlying fluid of the film was undoubtedly less active than the dilute control solution in which there was no loss of potency. Therefore, there is no evidence that the postulated active fragments of the native hormone had separated from the inactive film and were dissolved in the underlying fluid.

In the case of crystalline insulin the results are strikingly different from those obtained with the pituitary hormones. As can be seen from Table 4 no loss of activity occurred owing to unfolding.

or non-acidified material. These facts point against a reversible activation-inactivation process.

SEROLOGICAL ACTIVITY

From previous work (Rothen and Landsteiner, 1939) we know that ovalbumin films 7 Å thick had not lost their property to combine specifically with antibodies of homologous sera. Therefore experiments were undertaken with some very active anti-metakestrin rabbit serum (Chow, 1941). We tried first to find out whether a film of metakestrin was still capable of reacting with homologous immune sera. Tests were made in the following way. After the film had stayed in the trough for a definite period, transfers were made to four chrome-plated

TABLE 4. EFFECT OF SPREADING ON THE BIOLOGICAL ACTIVITY OF INSULIN
(All assays were performed at 34.5° C.)

Experiment	Dosage		Number of mice in each group	Per cent mice exhibiting convulsions after injection of			
	Units	γ N/Kg.		Film		Native protein	
				At pH 5.6	Acidified	At pH 5.6	Acidified
A	1.2	7.0	20		90		80
	0.9	5.3	20		65		65
B	1.0	5.9	20	5	0	5	
	1.4	8.2	30	60	37	60	

slides simultaneously and the thickness was measured. A drop of homologous, heterologous,² or normal serum was deposited on a plate and smeared over the "useful" area. After 2 minutes which were found sufficient for the serological reaction to go to completion the plates were washed with saline solution, then with water, and the increase of thickness observed was a measure of the amount of material combined with the film. If the antigen in an unfolded state does not lose its specificity then an appreciable increase in thickness owing to the combined antibodies should occur when the plates were treated with a homologous serum; on the other hand, no increase or a small increase in thickness owing to some non-specific adsorption phenomenon should take place with normal or heterologous sera. This is exactly what happened. An average increase of 24 Å was observed when the plates were treated with homologous sera, as compared with an average increase of seven Å after treatment with heterologous or normal sera. The influence of the time elapsing between the initial spreading of the film and the transfer was also investigated. During these intervals the films were kept under zero pressure. Some of the results are presented in Table 5. From

results obtained with films which have stayed more than 5 or 10 minutes on the surface have no significance on account of contamination. However, our tests showed that even after 20 hours the results were not altered. 2) The specific reaction metakentrin-antibodies, was the same, as judged by the increase of thickness, whether one layer or two layers of metakentrin film were on the plates, whereas the non-specific reaction greatly diminished, and in some cases totally disappeared when two layers of metakentrin were used instead of one.

The following question arises, Would an extended antibody film react specifically with the corresponding antigen? Pauling (1940) in his recent consideration assumed that denatured antibodies would most likely lose their specific properties, since, according to his view, the specificity results from a specific folding, the polypeptide chain being the same for all antibodies. It might, of course, be argued that only a two-dimensional pattern is necessary for the reaction.

The preparation of purified antibodies is of importance to make experiments bearing on this question. If whole immune sera are used for making the film it might be that only a very small percentage

TABLE 5. REACTION OF FILM OF METAKENTRIN WITH IMMUNE, HETEROLOGOUS, AND NORMAL SERA

Metakentrin film, thickness in Å	Number of layers	Time between spreading and transfer, in minutes	Rabbit serum used	Increase in thickness in Å after serum treatment
8	1	15	Antiguinea pig blood	8
10	1	15	Antihorse albumin	9
8	1	25	Normal rabbit	7
7	1	25	Antimetakentrin B*	28
7	1	30	Antipneumococcus Type III	10
9	1	30	Antimetakentrin C*	24
20	2	30	Antimetakentrin C*	20
8	1	60	Antipneumococcus Type III	7
7	1	60	Antipneumococcus Type III	11
15	2	60	Antipneumococcus Type III	0
7	1	60	Antimetakentrin A*	17
20	2	60	Antimetakentrin A*	19
7	1	90	Antipneumococcus Type III	11
15	2	90	Antipneumococcus Type III	0
8	1	90	Antimetakentrin C*	25
—	2	90	Antimetakentrin C*	31
8	1	2 hrs.	Antimetakentrin D*	27
24	2	2 hrs.	Antimetakentrin D*	30
7	1	2 hrs.	Anti-lymphocytic choriomeningitis†	6
7	1	15 hrs.	Antirabies	11
16	2	15 hrs.	Antirabies	5
8	1	15 hrs.	Antimetakentrin D*	21
19	2	15 hrs.	Antimetakentrin D*	27

* Letters refer to different rabbit sera.

† Guinea pig serum.

all of our experiments we have arrived at the following conclusions. 1) The specific reaction of a film of metakentrin with homologous antibodies was not diminished after a stay of the film of 15 hours in the trough. In some quarters it is believed that

² We are indebted to Dr. K. Landsteiner and to Dr. J. Casals for the heterologous sera.

of the area originates from the antibodies, the main area being formed by the albumin and globulin constituents of the serum. In this case negative results would be inconclusive.

Since purified antimetakentrin antibody was not prepared, tests were made with films formed from whole immune sera, and also from the globulin-

antibody fraction of immune sera. The results were negative: On treating with a solution of metakentrin, plates on which a mixed extended film from an immune or from a heterologous serum had been transferred the increase in thickness observed was about the same in both cases ($\approx 18 \text{ \AA}$). However,³ purified pneumococcus antibodies have been prepared and the results showed that unfolding did not completely destroy their antibody properties. Films of unfolded pneumococcus antibodies Type I and III were capable of reacting specifically with homologous polysaccharides.

CONCLUSION

From the above evidence it appears that the biological activity of two pituitary hormones studied is dependent to a high degree on a definite folding of the polypeptide chain. The biological activity depending on a unique spatial configuration is markedly lowered by the loss of this configuration. In contrast, a specific folding is not necessary in the case of insulin. Also the property of an antigen to react with its antibodies is not lost by the unfolding process and conversely the unfolding of an antibody in some cases does not destroy all of its specific property. The native configuration necessary for the biological activity of the pituitary gonadotropic hormone, metakentrin, is not essential for an antigen-antibody reaction. The "rule of parsimony" is in favor of such a state of affairs. It should be less effort to build up an antibody which does not require for its specificity a unique and complicated steric structure. A two-dimensional pattern seems to be a sufficient condition.

SUMMARY

By the unfolding process, the activity of insulin is unimpaired whereas that of the oxytocic-pressor hormone of the posterior pituitary, and of metakentrin, a gonadotropic pituitary hormone, is markedly reduced or destroyed.

Films of completely unfolded molecules of metakentrin, seven to nine \AA thick, have not lost their specific property to combine with homologous antibodies.

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³ An article, in collaboration with Dr. Landsteiner, describing the work in detail is in preparation.

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DISCUSSION

WRINCH: I am very much interested in the results relating to insulin films. In a number of respects, insulin is different from the majority of native proteins. Thus at the beginning of this work on protein films in 1936, it was noticed that many proteins, when spread in a monolayer and compressed in one direction, can be made to form fibrils normal to this direction. With insulin this did not prove possible. It was suggested that this may mean that the insulin molecule maintains intact, certain areas of its fabric structure even when the native molecule is ripped open to form a monolayer. That this may be the correct interpretation is suggested also by the very high content of leucine in insulin, amounting to about one in three of all the constituent residues. For the arrangement of such residues in the native protein structure in such a way that stabilizing hydrocarbon neighborhoods are formed (after the manner of the acetamide crystal) would allow also the maintenance intact of such neighborhoods (associated with peptide rings perhaps still in a highly organized multiply-connected atomic network) in the monolayer form. This stabilizing influence is also seen in the marked resistance to denaturation exhibited by insulin and also in the fact that insulin can form crystals with a low content of water molecules. All these rather special characteristics of insulin thus seem to tie in together and to offer an interpretation of Rothen's finding that insulin does not lose its biological activity on spreading. The point of view that biological activity in many cases may depend upon the arrangement of certain R-groups in the protein molecule in a definite spatial pattern (due to the fixed pattern of their C roots in the protein fabric) has been used by Marrack to explain the specificity of antigen-antibody complexes in immunology. It would seem that exactly the same picture is required by Rothen's insulin experiments.

It is also of interest that Rothen finds a standard thickness of 7 to 10 \AA for his monolayers. It must however be emphasized that such measurements give no reason for the assumption of polypeptide chains. A structure such as I have suggested for the insulin case, consisting of polymerized fabric fragments, would of course require a thickness of just the same order. In view of the very considerable body of data which suggest that biological specificities are asso-

ciated with rigid, multiply-connected atomic groupings, it seems important not to overlook this fact.

This type of monolayer structure also seems to be indicated by the interesting result that metakentrin does not lose its power to form specific complexes of the antibody-antigen type when in monolayer form. It is to be presumed here also that certain areas of the original fabric are left unchanged in the monolayer form.

ROTHEN: I agree with Dr. Wrinch in the case of insulin. The only explanation we can think of now is that part of a two-dimensional pattern is all that is required for its activity; the whole native pattern is not needed. Another possibility for which there is no experimental evidence is that the activity of insulin is located in a small prosthetic group unaffected by unfolding.

GREENSTEIN: When we denatured insulin with urea or guanidine HCl, the material opened up and disulphide groups appeared. The protein was still completely active. Substantially this is in agreement with Dr. Rothen's results.

STANLEY: What happens when you apply antibody at different concentrations to the film of antigen? Was the increase in thickness dependent upon the concentration?

ROTHEN: Since we did not prepare some purified metakentrin antibody we did not attempt to find out what relation there was between the concentration of the antibodies in the antiserum and the increase in thickness observed after the plates were treated by the antiserum. However, no appreciable difference in increase in thickness could be observed when an immune serum diluted 10 to 20 times with saline solution was used instead of the undiluted one. The only difference was that the time needed for the serological reaction to go to completion was greater than two minutes in the case of the diluted serum.

ANSON: As Dr. Rothen has realized, so long as a biological test for activity is used, a substance which is active after being spread may be active because the change brought about in spreading is reversed during the activity test, and a substance which is inactive after being spread may be inactive because it is insoluble under the testing conditions. The possibility of these complications cannot be avoided when hormones are used. Hormones, therefore, do not seem to be ideal material for the study of the effect of spreading on activity. Have you tried other

substances on which suitable tests can be made while the substances are spread?

ROTHEN: With Landsteiner, I have carried out some similar experiments with films of ovalbumin, horse serum albumin and horse serum globulin. Films of these substances seven to nine Å thick were capable of reacting specifically with homologous antibodies. However, films of globulin had interesting properties. They lost their specificity after staying a few hours on the surface of the trough. Something happened in the two-dimensional fabric. If the completely extended film, soon after formation, was compressed to 15 dynes or so its specificity was kept intact for at least 15 hours. But if decompressed it lost its specificity at about the same rate as a fresh film.

ANSON: Have you tried other substances, such as enzymes?

ROTHEN: Not yet.

CHOW: The term "Metakentrin" may not be familiar to all members of this audience. It was coined to refer to a specific pure pituitary gonadotropic hormone extracted from hog glands. Impure preparations of this hormone have been called luteinizing hormone or interstitial cell stimulating hormone. A pure metakentrin from sheep pituitaries has been isolated by Li, Simpson and Evans; its principal biological effects resemble those of hog metakentrin from which it can be differentiated serologically. The hormone, which can be satisfactorily assayed in hypophysectomized immature rats, stimulates the testicular interstitial cells whose secretion then maintains or stimulates male accessory organs such as the anterior prostate. Weight changes of the anterior prostate are the basis of our assay.

Comparisons of the effect of spreading insulin or posterior-lobe protein on biological activity are of interest because, although the insulin molecule is the larger and has been considered to exert its typical biological effect only when intact, yet the hyperglycemic action of the hormone is not demonstrably affected when its native configuration is destroyed by spreading it as a film. On the other hand, although the posterior-lobe hormone appears to be the parent of two active units (vasopressin and oxytocin) with molecular weights possibly of 1000 each, yet spreading of the parent protein is accompanied by marked loss of biological potency without evidence of retention of activity either of or by the two postulated active units or fragments.

SOME OBSERVATIONS ON PROTEIN FOLDING AND UNFOLDING

ALFRED E. MIRSKY

SPECIFIC PROPERTIES OF PROTEINS

The highly specific properties attributed to the gene direct our attention to the specific properties of proteins. The wonderful specificity exhibited by the respiratory proteins (hemoglobin and hemocyanin), enzymatic proteins and antibodies suggests that the specific properties of genes are in part due to their constituent proteins. In this paper some of the factors concerning protein specificity are considered. It will be seen that an investigation of the first steps in the destruction of the specific properties of a protein throws some light on the configuration of the native protein molecule.

The instability of native proteins has always been one of their most striking characteristics. Proteins can be denatured by many different agents—heat, acid, alkali, alcohol, urea, etc. Years ago, in experiments on egg albumin, it was shown that the change in a native protein known as denaturation can be defined by a marked change in solubility, the denatured protein being insoluble at its iso-electric point under conditions under which the original native protein is soluble (Chick and Martin, 1910). The same agents that denature a protein such as egg albumin, also denature hemoglobin, the enzymatic proteins and antibodies, denaturation in all cases being defined by change in solubility. The characteristic specific properties of these proteins are destroyed by the denaturing agents at the same time that the change in solubility is produced (Anson and Mirsky, 1930; Northrop, 1939). Loss of solubility and loss of specific properties appear to be different aspects of the same underlying process. This correlation is emphasized by the fact that when the denaturation of hemoglobin, trypsin, chymotrypsin, and pepsinogen is reversed, the unique properties of these native proteins are restored at the same time that they recover their original solubilities. The loss of unique properties in denaturation suggests that the unique configuration of the native protein is lost, and this is borne out by the large increase in entropy known to take place in denaturation, indicating that for a denatured protein there is a multitude of available configurations (Mirsky and Pauling, 1936).

It has been suggested that in denaturation the configuration of a protein molecule becomes more extended and open (Mirsky and Pauling, 1936; Astbury, Dickinson and Bailey, 1935; Neurath and Saum, 1939; Bull, 1940). Further evidence that denaturation is an "unfolding" process is given in this paper. In the new experiments measurements of sulfhydryl (SH) groups of egg albumin are com-

bined with observations on the size and shape of the protein molecule under different conditions.

PROTEIN SH GROUPS

SH groups become detectable as a result of denaturation. There are similar changes in other groups—S-S groups, the phenolic groups of tyrosine, and peptide bonds (Mirsky, 1938; Lineweaver and Hoover, 1941). A systematic investigation of the effect of denaturation on activities of protein groups has yet to be made. SH groups have been most frequently studied so far because relatively simple methods for estimating them and a color test for detecting them have been available. In the present investigation SH groups are estimated by means of their reaction with ferricyanide, the quantity of ferrocyanide formed being taken as a measure of the number of reactive SH groups. Ferricyanide does not react with native egg albumin. When egg albumin is placed in solutions of urea, guanidine hydrochloride, and Duponol sufficiently concentrated to denature the protein, its SH groups react at once with ferricyanide. In the presence of any one of these three denaturing agents, the same number of SH groups is found in egg albumin (equivalent to a cysteine content of 0.97%) (Mirsky, in press). In each case the appearance of SH groups is correlated with the formation of insoluble denatured protein, although it should be noted that SH groups are estimated while the denatured protein is dissolved in the denaturing agent, and the quantity of insoluble protein is determined after removal of the denaturing agent. As denaturation proceeds, it can be shown that in the fraction of protein with altered solubility SH groups are reactive, whereas in the fraction with solubility unchanged no SH groups are detectable. Appearance of SH groups and alteration of solubility seem to be integral parts of the same protein transformation. An understanding of why SH groups appear may, accordingly, be expected to throw light on the whole process of protein denaturation.

One explanation that has been suggested for the appearance of SH groups is that when the configuration of the protein molecule becomes more extended and open, reagents such as ferricyanide are able to reach groups that are located within the compact, native protein molecule (Mirsky and Pauling, 1936). In the native protein, according to this view, these groups lie behind a barrier formed by the network of atoms on the surface of the molecule. Another explanation that has been advanced is that SH groups are not present in native egg albumin, but

are actually formed during the denaturation process (Linderstrøm-Lang and Jacobsen, 1931). It is not unlikely that each explanation will prove to have some validity.

PROTEIN FILMS

A first step in the search for a relationship between protein unfolding and appearance of SH groups is to spread the protein at an interface and examine its SH groups while in this state. A film of egg albumin is only about 9 A. U. thick, much thinner than a molecule of egg albumin in solution, an ellipsoid with axes of 91 and 32 A. U. The film of egg albumin is so thin that it is unlikely there would be a barrier between any SH groups in it and ferricyanide in the surrounding medium. Ferricyanide does, in fact, react with films of egg albumin, and the number of SH groups is found to be 0.97 percent, the same as in egg albumin denatured by urea, guanidine hydrochloride or Duponol (Mirsky, *in press*). Spread proteins resemble denatured proteins in two other respects: they are insoluble and they have lost some, at least, of their specific properties (Rothen et al, 1941).

DENATURATION BY UREA¹

The appearance of SH groups in egg albumin which unfolds to form a film suggests that when SH groups are caused to appear by a denaturing agent such as urea, the protein unfolds even though it remains dissolved in the urea solution. Measurements (carried out in collaboration with Dr. Alexandre Rothen) of the sedimentation and diffusion constants of egg albumin denatured in concentrated urea solution show that denaturation does not change the size of the albumin molecule, but does change its shape. The denatured protein molecule has a dissymmetry constant of 4.5, much greater than has been observed for any native protein, and clearly indicating that denaturation results in an extraordinary increase in molecular dissymmetry.

When films of egg albumin are formed and when egg albumin is denatured by urea, SH groups appear as the protein molecule unfolds. Under certain conditions some of these groups disappear. (In these instances not due to oxidation.) If the SH groups of a protein film are estimated by their reaction with ferricyanide only after many films have been clumped together, it is found that the ferricyanide, though present in excess, is reduced very sluggishly and incompletely, reacting after 20 hours with only 55 percent of the SH groups present at the time the films are first formed. Apparently the clumping together of protein films covers up SH groups that were uncovered when the native protein unfolded.

A similar change takes place in a solution of egg albumin that has been denatured by concentrated urea, if the urea is subsequently diluted. Whereas

in concentrated urea the reaction between SH groups and ferricyanide is completed in one minute, if (after the protein has been in 9.5 M urea) water is added to reduce the urea concentration to 1.7 M, the reaction with ferricyanide is sluggish and even after 120 minutes, only 65 percent of the SH groups react. Even in 5 M urea, the reaction between protein SH groups and ferricyanide is slow and incomplete. If the original concentration of the urea is restored, the reactivity of SH groups is also restored. In none of these experiments do protein precipitates appear; the solutions remain clear.

Experiments with the ultra-centrifuge show why the protein SH groups are so unreactive in these solutions. It has been found that as the urea concentration is diminished, the albumin polymerizes. All the urea-diluted protein solutions are observed to be polydisperse in the ultra-centrifuge, and the more the urea is diluted, the larger is the average size of the protein particles. In 9.5 M urea the particle, though elongated, is of the same size as that of native egg albumin. When this solution is diluted so that the urea concentration is 6 M, the average particle size doubles; dilution to 4 M increases the average size still more, and renders the particle more polydisperse. Restoring the urea concentration to 9.5 M depolymerizes the albumin. Comparing sedimentation and SH activity experiments, it is clear that polymerization is correlated with inactivity of SH groups. Ferricyanide is either unable to reach SH groups within the protein aggregates, or it penetrates into them slowly.

Lowering the temperature has the unexpected effect of *increasing* the rate at which SH groups of denatured egg albumin in urea react with ferricyanide. This curious temperature effect suggested that perhaps lowering the temperature tends to break up the protein aggregates that form when water is added to a urea-denatured egg albumin solution. Experiments with the ultra-centrifuge demonstrate that lowering the temperature does indeed diminish the size of such aggregates.

HEAT DENATURED EGG ALBUMIN

When egg albumin is denatured by heat, the number of SH groups liberated is less than the number found in egg albumin denatured by urea, guanidine hydrochloride or Duponol (0.55% as against 0.97%) (Mirsky and Anson, 1935; Todrick and Walker, 1937; Kuhn and Desnuelle, 1938; Greenstein, 1938). This result for heat denatured egg albumin is obtained even if the protein is heated in the absence of salt and at a pH sufficiently removed from the isoelectric point so that the albumin remains in solution (Anson, 1939; Mirsky, *unpub.*). The number of SH groups found in heat denatured egg albumin is less than that found in albumin in concentrated urea, but it is the same as that found in a urea denatured albumin solution in which the urea has been diluted by addition of water. When

¹ The experimental results referred to have not yet been published.

urea is the denaturing agent, removal of the agent (dilution of the urea) causes some SH groups to disappear. Is it not possible that when heat is the denaturing agent, removal of the agent (cooling the solution) also causes disappearance of SH groups? With this possibility in mind, experiments were done in which ferricyanide was present while the albumin was being denatured by heat. Under these conditions the same number of SH groups is found as in the presence of urea or guanidine hydrochloride. It was also found, by using the ultra-centrifuge, that solutions of heat denatured egg albumin are aggregated to about the same extent as are solutions of albumin denatured by urea and then subsequently diluted with water. In heat denaturation, then, there is an unfolding sufficient to uncover all SH groups, followed by a polymerization which covers some of the exposed groups.

In egg albumin, denaturation causes an unfolding of the protein molecule with liberation of SH groups; withdrawal of the denaturing agent is accompanied by protein aggregation and disappearance of some SH groups. There are proteins (hemoglobin, serum albumin, trypsin, chymotrypsin, pepsinogen) in which withdrawal of the denaturing agent leads to reversal of denaturation. In these proteins denaturation probably is an unfolding process, much as it is in egg albumin, and reversal would, accordingly, mean that refolding takes place. May it not be that the polymerization of denatured egg albumin observed in the ultra-centrifuge should also be considered to be a folding process, one in which several molecules "fold" on each other? According to this view, folding may take place to restore a unique configuration or it may take place in a more or less random manner, as in egg albumin. It is possible that denatured egg albumin would fold in a less random manner if the denatured protein were in a different chemical environment. Our knowledge of the denaturation of various proteins indicates that when a protein becomes unfolded, it will fold again or polymerize as soon as the agent causing the unfolding is removed.

A word, finally, about the gene. The specific properties attributed to the gene lead us to suppose that one of its constituents must be a protein that, at least at certain times, possesses a folded configuration. Other components of the gene are desoxyribose nucleic acid and relatively simple basic proteins, the protamines and histones. Our knowledge of the gene will be extended when we know more about the interactions of these components.

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DISCUSSION

FANKUCHEN: How do you know that the resulting product of denaturation was elongated from the f/f_0 ratio? Nothing in the theory gives this interpretation preference over that of flat disks. The interpretation of flat disks suggests the possibility that the unfolding may be into two-dimensional fabrics, perhaps similar to the fabrics that Dr. Wrinch uses for her cyclol models. If the protein degenerates into fabrics, then regeneration, which must occur for reversible denaturation, might possibly be easier than if the denatured product were an elongated polypeptide chain.

It has been assumed that proteins are ellipsoidal. It is now shown that this assumption is not warranted. In a recent paper, Oncley shows clearly that with a given f/f_0 ratio one can obtain all phases from elongated ellipsoids to flattened ellipsoids depending on the state of hydration.

MIRSKY: In calculating the shape of the protein molecule from the f/f_0 ratio certain assumptions are made, as Dr. Fankuchen has pointed out. I stated in my talk that it is necessary to make certain assumptions and that in doing so I was following the procedure of Svedberg and his collaborators. There is at present nothing to add to the discussion of this question given by Svedberg and Pedersen. They have pointed out that if the protein molecule is ellipsoidal there may be no way of telling from the f/f_0 ratio whether it is an oblong or oblate ellipsoid. They have also shown how significant a factor hydration may be. It should be stated that even if the molecule of denatured egg albumin has the shape of an oblate ellipsoid, it is most unlikely that it has a fabric similar to that used in the cyclol model. The important point about our results is that they unequivocally show that unfolding takes place in denaturation. Hydration can affect the increased asymmetry calculated from the f/f_0 ratio to only a slight extent.

In the paper by Oncley to which Fankuchen refers (ANNALS OF THE NEW YORK ACADEMY OF

SCIENCES 41:121, 1941) the treatment in all essentials follows that given by Svedberg and Pedersen. After allowing for hydration Oncley concludes that most of the protein molecules he discusses are in fact ellipsoidal.

GREENSTEIN: On the basis of the sulphhydryl data, do all three denaturation agents unfold the proteins to an equal extent?

MIRSKY: In egg albumin all the denaturing agents examined produce the same number of SH groups. In so far as appearance of SH groups is an indication of protein unfolding, the different denaturing agents unfold egg albumin to the same extent. SH groups provide a useful guide for investigations of protein unfolding, but it would be folly to depend solely on estimates of activity of SH groups. We have constantly supplemented data concerning SH groups with sedimentation and viscosity data. The SH groups of egg albumin in saturated urea and in saturated urea diluted to a concentration of 6M react with ferricyanide in the same manner, and yet sedimentation and viscosity experiments clearly show a difference in the state of the protein.

GREENSTEIN: Your definition of unfolding denaturation must then depend on the type of agent you use.

MIRSKY: There is at present no need, it seems to me, to add to the definition of denaturation I have given. The loss in solubility and loss of specific properties are the changes in a protein produced by all denaturing agents. Denaturation should be defined by these characteristics common to all forms of denaturation. It now appears that in those forms of denaturation which have been carefully studied unfolding takes place. The next step was to show that unfolded proteins tend to refold and aggregate and so it became clear that many of the differences observed in denaturation are due to what takes place *after* unfolding. There is at present no information concerning differences in the unfolding process itself.

GREENSTEIN: But I would like to emphasize that a definition of denaturation must be based on the denaturant and the particular protein used, as well as the specific property of the protein investigated. Loss in solubility is not at all characteristic of all forms of denaturation nor loss in specific activity.

WRINCH: The picture which Dr. Mirsky has given of the denaturation of egg albumin interests me in several ways, not least because it would seem so clearly to indicate a cage structure as predicted by the fabric theory of protein structure. If SH groups are present but are not accessible to reagents, it would seem to follow that they lie within an atomic framework which the reagent cannot penetrate. This interpretation which would seem to be required by Dr. Mirsky's data is specially relevant in connection with the "enol" fabric suggested in 1940. This fabric, unlike the original "lactim" fabric, places some of its R-groups with their C_β atoms within the atomic shell and some with their C_β atoms without. In any case it would be inter-

esting to know in what ways the inaccessibility of the SH groups can be interpreted except in terms of a closed hollow structure, especially in view of the fact that this working hypothesis regarding native protein structure gives a precise formulation to Dr. Mirsky's picture of "unfolding" which is consistent with immunological, physicochemical, and crystallographic data regarding native protein structures.

The second point is in connection with the nature of the links or bonds between the residues in the protein which differentiate the native form from the fully denatured forms. It is my belief that this link may be of a different kind from the links which the protein material makes after denaturation. For the interlinking of denatured proteins, whether this be of inter- or intra-molecular type, the hydrogen bond gives a plausible picture, especially in view of the high degree of insolubility in water of even the lower glycine peptides where hydrogen bonds must presumably be postulated. This suggestion fits also with the data regarding nylon's structure, presented by Dr. Mark, which even in its unstretched state gives by no means a typical "amorphous" X-ray picture. For the native protein, the situation is of course entirely different. Here the polycondensation structure of amino acids is of a very different type, judging by the solubility data regarding even the glycine peptides. Apparently the native protein, e.g., egg albumin, solves the problem of remaining soluble in water. This means that the residues are so arranged that they cannot, as in the case of, say, pentaglycine, form inter- or intra-bonds to such an extent as to make the resulting structure insoluble in water. It must be remembered that it is the general rule in structure chemistry that a structure with polar groups which forms hydrogen bonds thereby reduces its affinity for water molecules and thereby its solubility in water. This fact to which attention has often been called is perhaps best exemplified by the three nitrophenols, ortho, meta and para. It would seem to follow that the bond which makes possible the highly soluble native structure of egg albumin is not one which reduces its solubility in water, i.e., that it is not a hydrogen bridge. If this is so, it must be a covalent bond, and as such of wholly different character from the interlinking exhibited by denatured proteins.

If Dr. Mirsky confines his consideration of possible covalent bonds in native proteins to SS bonds, or even includes also other covalent bonds between R-groups, it is difficult to see how he can give any picture of the essential native protein character, which must characterize proteins in general. Such R-group covalent bonds must vary very much in efficiency for the building of the coherent structure of protein megamolecules, owing to the very different complements of such groupings in different substances, to none of which the name of "native protein" can be denied. All the data seem to point definitely to the view that if, as seems likely in view of arguments put forward on many previous occasions, the native protein bond is covalent in char-

acter, then this is a covalent bond between the skeletal atoms of the constituent residues. In this event there seems little choice but to postulate the replacement of the $>C=O$ formulation of some of the native protein residue by $>C(OH)$, the question as to whether the complete residue should be written in the form $>N-CHR-C(OH)<$ as in the "lactim" fabric or in the form $NH-CR-C(OH)<$ as in the "enol" fabric being left for further consideration. As we have pointed out, the stereochemistry of the residues would then seem to favor the enol form, if as Dr. Mirsky's results suggest some of the R-groups lie within the closed fabric structure.

MIRSKY: Dr. Wrinch says that "if SH groups are present but are not accessible to reagents it would seem to follow that they lie within an atomic framework which the reagents cannot penetrate." This is, of course, the picture of the native egg albumin molecule that I have given. Dr. Wrinch believes that this picture clearly indicates a "cage structure." I would say that the atoms in any closely folded arrangement of peptide chains would act as a barrier to the passage of a relatively large ion such as ferricyanide into the interior of the protein molecule. The experiments on inaccessible groups in native proteins do not favor the "cage structure" over other folded configurations.

Experiments on the action of urea on both native and denatured egg albumin indicate that when urea is withdrawn after denaturation has taken place, bonds form between different molecules of denatured, proteins, and that these bonds bear some resemblance to the bonds that hold the peptide chains of native egg albumin in their folded configuration. Dr. Wrinch prefers to suppose that links of a different character are responsible for the configuration of a native protein. She says that the loss of solubility observed in denaturation argues against the view that I have suggested. The insolubility of denatured protein presents no difficulty. When egg albumin is denatured by urea many bonds are broken and when the urea is removed these bonds tend to form again in a random manner so that different protein molecules are linked together. As more and more urea is withdrawn, the complexes linked together get larger until they finally form visible, insoluble particles. In the native protein these bonds are formed intra-molecularly so that massive, insoluble aggregates are not formed. Another factor that may influence the solubility of the protein is the distribution of hydrophilic and hydrophobic groups. It is possible that hydrophobic groups lying in the interior of the native protein molecule became exposed during the unfolding that occurs during denaturation and so render the denatured protein less soluble than the native.

WRINCH: Dr. Mirsky's additional comments on my views serve to underline several interesting conclusions. 1) Unless he is deliberately misunderstood, the main outlines of his conception of the structure of the native protein can be superposed upon—even

identified with—views which others have been expressing for several years past (Wrinch, *Proc. Roy. Soc. London* 160A, 59, 1937: 161A, 505, 1937 et seq.; Langmuir, *Proc. Phys. Soc. London* 51, 592, 1939). Dr. Mirsky thinks in terms of a "closely folded arrangement of peptide chains" which acts as "a barrier to the passage of a relatively large ion . . . into the interior." I have suggested a lacunated atomic fabric, folded round to form a closed structure (*Cold Spring Harbor Symposium on Quantitative Biology* 6, 122, 1938 etc.). Pending the discovery of a more agreeable synonym, we may continue to call such a structure a cage. It seems hardly surprising that two conceptions, differing only in name, account equally satisfactorily for the decreased solubility of denatured proteins or that "experiments on inaccessible groups of native proteins do not favor the cage structure over other folded configurations." 2) In the matter of the position of hydrophobic groups in native proteins, it is interesting to notice that Dr. Mirsky finds that his experiments favor the view previously proposed on several occasions (e.g. Langmuir, *Cold Spring Harbor Symposium on Quantitative Biology* 6, 173, 1938; Wrinch, *Phil. Mag.* 30, 64, 1940:31, 177, 1941) that such groups may lie in the interior, shielded by the atomic fabric of the cage from a watery external world. 3) It appears from Dr. Mirsky's supplementary remarks that his experiments give little or no information regarding the nature of the bonds or links which hold the residues in their unique configurations in the native protein. There seems then little to add to my previous argument (see above) that, since such bonds or links must be such as to account for the high solubility in water of (e.g.) the native egg albumin molecule, something other than a hydrogen bridge, say a covalent bond, must be postulated to distinguish the disordered linear peptides (which he assumes to be present in denatured proteins) from the perfectly ordered polycondensations of amino acid and imino acid molecules which make up the highly organized native protein molecule.

ROTHEN: You can form films of egg albumin with denatured albumin as well as the native protein.

WRINCH: Isn't denatured albumin still undetermined as to structure? The native protein is one of much more difficulty.

GREENSTEIN: It may be pointed out that these reagents cause not only unfolding but may also produce disaggregation; for example, in the case of myosin or tobacco mosaic virus, where you might have either unfolding or depolymerization or both.

CHILD: Are there any properties which the denaturing agents have in common?

MIRSKY: All form H bonds readily.

UBER: The inactivation of trypsin by ultraviolet radiation indicates that only a single quantum is required for the inactivation of a molecule.

THE STATE OF THE PROTEINS IN ANIMALS AS REVEALED BY THE USE OF ISOTOPES

D. RITTENBERG

The introduction of the isotope technique to biochemistry has developed a new method of attacking many old problems. With this tool it is now possible to study intermediary metabolism in the normal intact organism and to observe the rates of the chemical reactions involved without disturbing the finely balanced systems.

Hevesey in 1923 first made use of a radioactive isotope to study lead metabolism. Shortly after Urey made available the heavy isotope of hydrogen, Dr. Schoenheimer and I began the study of fat metabolism with this new tool. With the preparation of the heavy isotope of nitrogen this work was extended in collaboration with Dr. Ratner and Dr. Keston to the study of protein metabolism.

The living organism cannot detect variations of isotope concentration of the elements composing a compound for such changes do not lead to appreciable changes in chemical or physical properties. There are now available two types of isotopes, the stable and the radioactive. Each has its particular advantages and disadvantages. For the investigation of organic compounds the stable ones seem most suitable at present. In any case most work on the proteins has been carried out with the stable isotopes of hydrogen and nitrogen.

If a sample of glycine is prepared in which the normal isotope abundance of nitrogen is increased, the compound is chemically and physiologically identical with the normal glycine, but can be detected and measured even when mixed with large amounts of glycine present in the organism. If such a compound is fed to an animal, the rate at which it is incorporated into the protein and at which the nitrogen of the amino group is transferred to other compounds can be measured. Such investigations have thrown a new light on the great chemical reactivity of compounds in the living cell.

That the cell has the capability of carrying out many complex chemical reactions with great velocity is shown by almost every type of investigation employed in the field of biochemistry. In the intact animal the absolute rate of most of these reactions was unknown. What was known was only the difference between the rates of the synthetic and degradative reactions. The composition of a mature animal is constant within rather narrow limits, but whether this constancy is due to a lack of chemical reactions or to the equality of the rates at which the cellular constituents are being synthesized and destroyed was quite obscure.

The living cell has no difficulty in synthesizing proteins and degrading them. It was generally assumed that in the mature animal both of these re-

actions were slowed down. The extent of the interaction of the dietary nitrogen with the tissue nitrogen was unknown. As the quantity of nitrogen excreted was closely parallel to that in the diet, it was believed that only a small amount of the daily protein intake was used for making good the "wear and tear" of the organism while the remainder was oxidized.

The results obtained from feeding labeled amino acids require a modification of this view. In a typical experiment (Schoenheimer, Ratner and Rittenberg, 1939) full grown adult rats were kept for three days on an adequate diet to which had been added 215 mg. of labeled *l*(-)-leucine per rat per day. The α -amino group of the leucine had been labeled with heavy nitrogen, N¹⁵. At the end of this experimental period the animals were killed and the N¹⁵ distribution determined. In Table 1 is given the isotope balance of the experiment.

TABLE 1. BALANCE OF NITROGEN ISOTOPE AFTER FEEDING *l*-LEUCINE

		Fraction of N ¹⁵ administered
Excreta	Feces	percent 2.1
	Urine	27.6
Animal body	Non-protein N	7.8
	Protein N	57.5

While the total amount of excreted nitrogen was equal to that in the diet, less than one-third of the marked leucine nitrogen was recovered in the excreta. More than half of the nitrogen of the leucine fed was present in the proteins. As what had been offered was a free amino acid, and what had been analysed were proteins, it is apparent that what we observed here is due not to a simple mixing process, but to extensive chemical reactions. The process must be so fast that half of the dietary leucine N can be incorporated into the protein. The rate of transport of leucine N into the proteins is faster than its conversion to urea.

By examination of proteins of different organs, one finds that they do not take up equal concentrations of the labeled nitrogen. In Table 2 are shown the values obtained in the *l*-leucine experiment. The blood proteins have taken up the labeled nitrogen most rapidly, the skin most slowly. Intestinal wall, kidney, spleen and liver follow the blood proteins in activity. Similar results are obtained after

TABLE 2. N^{15} CONCENTRATION IN THE PROTEINS OF RATS FED ISOTOPIC L-LEUCINE
The values are calculated for an N^{15} concentration of 100 atom percent in the leucine added to the diet.

Organ	N^{15} concentration <i>atom percent excess</i>
Blood plasma	1.65
Erythrocytes	0.29
Liver	0.93
Intestinal wall	1.48
Kidney	1.36
Heart	0.89
Spleen	1.10
Testes	0.76
Skin	0.18
Muscle	0.31

feeding tyrosine and glycine. The N^{15} content of the proteins is not due solely to the introduction of the fed amino acid since, as I shall show later, the labeled nitrogen can be found in almost all of the amino acids of the protein. The figures in the last column give the percent of the total protein N which was derived during the experimental period from the labeled leucine nitrogen. Thus 1.65 percent of all the nitrogen of the blood protein has been derived in this three-day period from the 69 mg. of leucine nitrogen added to the diet. The figures in Table 2 may seem low, but it must be remembered that if the labeled nitrogen had been evenly distributed over all the proteins, there could have been no more than a 1 percent replacement.

These organ proteins are not well characterized compounds but mixtures of all the proteins of the particular cell system investigated. In order to work with a more closely defined system, some investigations have recently been carried out in collaboration with Dr. M. Heidelberger (unpublished data) on antibodies. A rabbit was immunized to Type III pneumococcus. Ten days after the last injection of antigen a small amount of glycine labeled with N^{15} was added to its diet. Daily thereafter samples of blood were taken and analysed for total antibody content, and the N^{15} content of the antibody. Simultaneously the N^{15} content of the remaining blood proteins was determined. During the three-day feeding period the N^{15} concentration of the antibody and of the blood proteins increased by about the same amount. After the removal of isotopic glycine from the diet the N^{15} content of both protein fractions decreased. The N^{15} content of the antibody fell to one-half its maximum value in about 12 days. Both the increase of N^{15} concentration in the antibody during the feeding period as well as the decrease in the following period are the result of the formation of new antibody; in the one case with the labeled nitrogen, and in the other with normal nitrogen.

The quantitative antibody determinations showed that during this period the amount of antibody in the serum steadily decreased. We here observe syn-

thesis of a specific protein during the time when the total amount of this protein is decreasing; the degradative rate is faster than the synthetic rate.

The metabolic activity of the tripeptide glutathione, GSH (glutamyl cystinyl glycine), has recently been studied in collaboration with Dr. Waelsch with labeled glycine (Waelsch and Rittenberg, 1941). It was found two and a half hours after feeding glycine to rabbits that 4.6 percent of the liver GSH nitrogen was derived from the glycine fed. Not all the labeled nitrogen was present in the glycine portion of GSH, but about one-third was in the glutamic acid and cysteine combined. In Table 3 is shown the distribution of

TABLE 3. N^{15} CONCENTRATION IN GLUTATHIONE AND ITS AMINO ACIDS AFTER FEEDING ISOTOPIC GLYCINE
The values are calculated for an N^{15} concentration of 100 atom percent in the glycine fed.

Substance	N^{15} concentration <i>atom percent excess</i>
Glutathione	4.6
Glutathione-glycine	9.0
Glutathione-glutamic acid	2.6
Glutathione-cysteine	2.2

one typical experiment. From these results it may be seen that four percent of the glycine of glutathione has been hourly replaced by fed labeled glycine. In 14 hours half of this reaction will be completed. In all such calculations the results give minimal values, for in them we assume that what was available in the cell was only the labeled glycine. In actuality the material fed must be diluted with glycine liberated from the proteins.

There is some evidence from detoxication studies that the fed glycine is diluted by about a factor of three by normal glycine of the animal before it becomes available for chemical reactions. If glycine and benzoic acid are administered to either rats or rabbits, the benzoic acid is excreted conjugated with glycine. In such experiments the excreted hippuric acid contains about one-third of the isotope concentration of the fed material. This indicates that the concentration of the N^{15} in the isotopic glycine available to the cell for its metabolic reactions was diluted by a factor of three. If this dilution factor also existed in the GSH study, the time necessary for the substitution reaction to proceed half way to completion would be reduced to four hours. I cannot say whether the N^{15} content of the GSH is due to a complete resynthesis or merely to substitution of a single amino acid at a time. It would be difficult to formulate any mechanism by which labeled cysteine was incorporated in GSH without a complete synthesis.

The knowledge of the isotope concentration in the material available to the cells is the most pressing problem for the evaluation of rates of reaction from this type of experiment.

As far as the glycine figures are concerned, we primarily are measuring the rate of splitting and reformation of a peptide bond. While no definite information is available, it seems reasonable that this peptide bond is typical and it can be presumed that these data apply to proteins in general. The rate of this reaction will of course vary from protein to protein.

Not only does the use of labeled nitrogen in amino acids simplify the determination of the rate of its introduction into peptides and proteins, but we can simultaneously follow the transfer of nitrogen to other amino acids and related compounds. As can be seen from Table 3, the feeding of glycine has given rise to labeled glutamic acid and cysteine. Glycine nitrogen has been employed in the synthesis of these two amino acids.

If we now return to a closer examination of the *l*(-)-leucine experiment, we can see more clearly the extent of the nitrogen transfer reactions. Leucine is known to be an essential amino acid for the rat from the experiments of Womack and Rose (1936). They have shown that *l*(-)-leucine can be substituted by α -hydroxy isocaproic and α -keto isocaproic acid. From this finding it can be concluded that only the carbon chain is essential and that the amino group is not. This phenomenon seems to be generally true, with but few exceptions, for most amino acids. The amino acids thus seem to have a two-fold pathway in the intermediary metabolism, one being taken by the amino group, the other by the carbon chain. In order to follow adequately the route taken by an amino acid two labels are necessary, one for the amino group, N^{15} , and one for the carbon chain, either H^2 or C^{13} .

We have, therefore, prepared leucine in which the carbon chain was labeled by substituting deuterium atoms for carbon bound hydrogen, and the amino group by heavy nitrogen. The optically inactive compound was resolved prior to feeding. The *l*(-)-leucine finally obtained contained 3.60 atom percent excess of D and 6.5 atom percent excess N^{15} , these being in the ratio of 100 to 182.

After feeding this compound for three days to rats, the animals were killed and from the proteins of the liver, intestinal tract and carcass several amino acids were isolated. The isotope concentrations in the liver constituents are given in Table 4.

In each protein the highest N^{15} concentration was found in the leucine. This seems quite reasonable as it was the amino acid fed. Nevertheless this assures us that it is the amino acid itself which is introduced into peptide linkage, and not some deaminized derivative.

From these figures it can be calculated that 8 percent of all the leucine nitrogen of the liver protein is derived from the labeled leucine added to the diet. As the diet contains an equal amount of normal leucine, about 16 percent of the liver leucine nitrogen had been derived from dietary leucine nitrogen in 3 days. Five percent of the leucine nitrogen of

TABLE 4. N^{15} CONCENTRATION IN PROTEIN CONSTITUENTS OF LIVERS OF RATS FED ISOTOPIC L-LEUCINE
These values are calculated for an N^{15} concentration of 100 atom percent excess in the leucine fed.

Substance	N^{15} concentration
	atom percent excess
Total liver protein	0.93
Amide nitrogen	0.78
Glycine	0.73
Tyrosine	0.51
Aspartic acid	1.16
Glutamic acid	1.85
Arginine	0.89
Lysine	0.06
Leucine	7.92

the liver protein is being replaced daily by dietary leucine nitrogen. As this leucine was also labeled with deuterium, we can calculate from the deuterium figures the introduction of the carbon chain of dietary leucine into the proteins. The deuterium content of the leucine isolated from the liver protein was 0.44 atom percent excess, while the material fed, corrected for dilution by the normal leucine of the diet, was 1.8 atom percent excess. This indicates that at least 24 percent of the leucine carbon chain in the protein of the liver has been derived from the dietary leucine (8 percent per day). If the dietary leucine had only been diluted with normal leucine of the protein, then the ratio of the deuterium concentration to that of the N^{15} concentration in the leucine isolated from the tissue would not change, though the absolute values would be lower. In fact, the D: N^{15} ratio changed from 100:182 in the fed material to 100:103 and 100:117 in the carcass and liver respectively. In addition to dilution of the isotopes, chemical reactions must have occurred by which more than one-third of the nitrogen in the original leucine has been removed and replaced by normal nitrogen from other sources. Leucine is being deaminated and reaminated at a rate as high as 10 percent per day in these tissues.

The nitrogen of the dietary leucine is found in the tissue proteins not only in the same amino acid but widely distributed over most of the other amino acids. Thus the labeled nitrogen is present in tyrosine, glycine, the amide groups, etc. This is not a one-way reaction, for when tyrosine or glycine are fed, their labeled nitrogen can be found in the leucine. This transfer of amino groups proceeds even though the total amount of each particular amino acid is constant. It is a general reaction true not only for leucine but also for many of the other constituents of the proteins.

One would at first call this a reversible reaction, were it not for the fact that the organism as a whole is not an equilibrium system. It can best be described as a system in a steady state. The composition is kept constant not because there are no reactions, or that the system has come to equilibrium, but because the rate of production of the

cellular constituents is equal to their rate of degradation. According to this view the composition of the cell is governed by the rates of chemical reactions.

Since the glutamic acid of the leucine experiment contained labeled nitrogen, it was an indication that the amino acid had been synthesized. This in spite of the fact that the organism was being supplied with relatively large amounts of glutamic acid in its diet. The synthesis of a substance while the organism is liberally supplied with it is a general phenomenon, observed in the metabolism of fatty acids as well as in the amino acids. The continuous synthesis of tyrosine from phenylalanine has been observed even though tyrosine is liberally supplied in the diet (Moss and Schoenheimer, 1940). This synthesis is readily understandable by the concept of the steady state. All the enzyme systems in the organism, whether synthetic or degradative, are actively at work. The systems involved in the synthesis of glutamic acid are active even though large amounts of glutamic acid are supplied to the organism. The degradative systems also are continuously active though the amount of substrate they decompose is probably dependent on the concentration of the substrate. On this hypothesis all the molecules, with but few exceptions, are steadily being degraded and resynthesized. The first insight into these rates was obtained in the field of fatty acids. Here it was found that in mice on a fat-free diet one-half of all the fatty acids were resynthesized in about seven days (Rittenberg and Schoenheimer, 1937). The rate of turnover of the amino acids in normal animals is a more complicated problem as one feeds amino acids all the time. Clearly the rate of turnover of the amino acids of a protein will among other things be dependent on the amount of the amino acid fed, and also the amount of other substances which can be converted into the particular amino acid. Under the conditions used in our laboratory there is some evidence that the glutamic acid of the liver proteins has practically been completely resynthesized, i.e., has at least acquired a new amino group, in less than three days.

Urea is the end-product of the nitrogen metabolism and is probably a representative sample of the metabolic nitrogen pool available for the average reaction. Since the glutamic acid accepts nitrogen not from one amino acid but from many, it is not to be expected that the glutamic acid will, when the steady state is attained, have the same isotope concentration, as the leucine fed. It will be much lower. It is a reasonable hypothesis that it will approximate that of the urea. In our experiments the concentration of N^{15} in the glutamic acid is more than three-fourths that of the excreted urea. This strongly suggests that the glutamic acid has come close to its maximum N^{15} concentration in three days.

In another type of experiment in our laboratory, we have come to a closer determination of the rate at which glutamic acid enters into the chemical reactions of the cell.

If a mouse is injected with heavy water so that its body fluids contain a high concentration of deuterium oxide, and the animal is given that concentration of heavy water to drink which will just maintain this deuterium content of its body fluids, then all the chemical reactions will take place in a medium of the same D_2O concentration. In these reactions, hydrogenations, hydrations, etc., deuterium may be taken up by the molecules into stable positions. By stable positions we mean those deuterium atoms attached to carbon bonds which cannot be removed by merely dissolving the compound in water or by boiling it in aqueous acid or alkali.

When such experiments are carried out with mice, some of the amino acids of the proteins take up deuterium (Foster, Rittenberg and Schoenheimer, 1938). These experiments were carried on for 10 days and for 63 days. Were the rate at which deuterium enters into amino acids a slow reaction, then the results of the 63 day experiment should be markedly higher than that of the 10 day experiment. In actuality they are almost the same. In leucine the deuterium content corresponded to a replacement of one hydrogen atom, in histidine to 1.5 atoms, in aspartic acid to one atom, in glutamic acid to 2.2 atoms, and in lysine to none. At the time these experiments were published, we stated that the deuterium content of these amino acids was probably the result of continuous deamination and reamination. If this replacement is due to deamination and reamination, then at least the α -carbon hydrogen must have been replaced by deuterium. While the compound is in the α -keto stage, further deuterium may be introduced by exchange reactions in the β -carbon atom.

As the 10 and 63 day experiments are closely the same, the rate of this reaction must be so fast that it is almost completed in less than 10 days. This can only mean that all the α -amino groups of the amino acids which can be replaced have been in 10 days. We have recently carried out in this laboratory some experiments which indicate that in the rat liver this replacement of carbon bound deuterium in glutamic acid is more than two-thirds completed in 24 hours.¹ If, as we assume, the reaction we observe is deamination and reamination, this reaction also has a half time of less than 24 hours. As it seems reasonable that this reaction takes place with free glutamic acid, the rate at which the amino acid enters and leaves the protein must be even faster.

When glutamic acid labeled with N^{15} is given to rats, a picture quite different from the other amino acids is observed.¹ The concentration of N^{15} in the glutamic acid is not much higher than that of the other amino acids. The isotope distribution in this case resembles that which is observed when ammonia is fed. In the three day experimental period the nitrogen of the glutamic acid has been almost completely detached and replaced from other

¹I am indebted to Doctors Schoenheimer and Ratner for permission to use these unpublished data.

sources. From these three experiments one can conclude that the reactions to which glutamic acid is subjected in the liver are so rapid that they are half completed in about one day.

This chemical lability is not restricted to glutamic acid, but seems to be a general property of most amino acids in the organism. It may be that the reactions which glutamic acid undergoes are more rapid than the other compounds, but in principle they are the same.

Similar metabolic activity has been shown by deuterio ornithine (Clutton, Schoenheimer and Rittenberg, 1940). Ornithine is not a constituent of proteins but is obtained as a degradation product from arginine. Krebs and Henseleit (1932) demonstrated that ornithine when added to liver slices increases the production of urea. It has been postulated that the reaction goes *via* the formation of arginine from ornithine, and the subsequent production of ornithine and urea by the action of arginase. This cycle is believed to occur while the compounds are in the free state.

After 10 days of feeding of mice with deuterio ornithine, there was isolated from the total proteins a deuterio arginine, demonstrating the conversion of ornithine to arginine. From the isotope concentration it could be calculated that about one percent of the total protein arginine had been synthesized daily from the ornithine fed. While this seems to be a relatively slow reaction, it must be remembered that in addition to the conversion, the arginine formed had to be transported from the liver to the various tissues, arginine already in the protein removed through opening of at least two peptide bonds, and finally the deuterio arginine introduced into the protein.

Besides the conversion to arginine some of the ornithine was converted into glutamic acid and proline, for these compounds when isolated contained deuterium (Rolloff, Ratner and Schoenheimer, 1940). Krebs on the basis of tissue slice experiments has advanced evidence that proline, ornithine and glutamic acid are metabolically interlinked (Krebs, 1939). The present experiments on normal animals demonstrate that these conversions and the introduction of the newly formed amino acids into proteins are a continuous process. It is most striking that the proline isolated contains one-third of the isotope concentration of the arginine. The rate of conversion of ornithine to proline must be of the same order of magnitude as its synthesis to arginine. These reactions, synthesis of proline and glutamic acid, take place in spite of the fact that the diet contains adequate amounts of these amino acids, again illustrating the fact that synthesis of a compound is not dependent on the occurrence or absence of this compound in the diet.

When *L*-leucine was fed to rats, the arginine isolated from the proteins contained labeled nitrogen. Degradation showed that the N^{15} was mainly in the amidine group. This is the result of the urea

cycle. As arginase is rather ineffective on arginine in the protein, this reaction probably took place while the amino acid was in the free state. If so, the arginine of the protein and the free arginine must rapidly interchange, as the N^{15} concentration of the amidine group is one-half of that of the urinary urea.

Schoenheimer and Bloch have prepared an arginine in which the amidine group was labeled by N^{15} , and have studied its reactions in rats (Bloch and Schoenheimer, 1941). As was expected, the urinary urea contained a high concentration of N^{15} . It was shown that the amidine group of arginine was transferred as a unit to glycine, and the resulting guanido acetic acid methylated to give creatine. The methyl groups for this reaction are derived from another amino acid. Du Vigneaud *et al* (1940) have prepared and fed a methionine in which the methyl group was labeled with deuterium. With this compound they have demonstrated the shift of the methyl group from methionine to creatine. These experiments have completely elucidated all the sources used for creatine formation, glycine, arginine and methionine. Methionine not only supplies methyl groups for creatine but also for choline.

The amino acid lysine is different in its reactions from all others we have investigated. In all experiments in which we have kept animals on a deuterium rich regime or in which labeled ammonia or amino acids have been fed, we have never found any of the isotype in the lysine of the proteins. We concluded from these facts that lysine was not involved in any reversible biological reaction involving the carbon chain. A close examination of this problem has been completed in our laboratory. Schoenheimer and Weissman have synthesized an *L*-lysine in which the carbon chain was labeled with deuterium and the α -amino group with N^{15} . The ratio of these isotope concentrations was 3.6 to 1. This material was added to the diet of rats and lysine subsequently isolated from the proteins of the animals. As the lysine isolated was a mixture of that deposited and the normal lysine already present, the isotope concentrations in the isolated lysine were lower than in the material fed. As stated before, such dilution will not alter the relative ratio of the isotope concentrations. This can only be effected if the labeled α -amino group is removed and replaced by normal nitrogen. As the ratio of the isotope concentrations of the isolated lysine was the same as that of the material fed this reaction could not have occurred. That lysine can be degraded is demonstrated by the finding of the labeled α -nitrogen in other amino acids. It differs therefore from other amino acids only in the fact that it cannot be resynthesized. Once lysine is converted to another compound, it is never converted back to lysine.

In all experiments carried out, whether with amino acids or ammonia, the highest concentration of N^{15} , with the exception of the amino acid fed, is in the glutamic acid. The significance of this is

as yet unknown, though it may be related to the presence of an enzyme system in the organism which can convert α -keto glutaric acid and ammonia to glutamic acid.

The viewpoint of our laboratory on the state of proteins in the animal can best be summarized by quoting from a recent paper (Schoenheimer, Ratner and Rittenberg, 1939): "It has been shown that nitrogenous groupings of tissue proteins are constantly involved in chemical reactions; peptide linkages open, the amino acids liberated mix with others of the same species of whatever source, diet or tissue. This mixture of amino acid molecules, while in the free state, takes part in a variety of chemical reactions: some reenter directly into vacant positions left open by the rupture of peptide linkages; others transfer their nitrogen to deaminated molecules to form new amino acids. These in turn continuously enter the same chemical cycles which render the source of the nitrogen indistinguishable. Some body constituents like glutamic and aspartic acids and some proteins like those of liver, serum, and other organs are more actively involved than others in this general metabolic mixing process. The excreted nitrogen may be considered as a part of the metabolic pool originating from interaction of dietary nitrogen with the relatively large quantities of reactive tissue nitrogen."

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DISCUSSION

ROTHEN: Where do you find the N^{15} if you feed an animal with d-leucine instead of l-leucine?

RITTENBERG: About 60 percent of it is excreted as ammonia and urea. The rest you can find in all

the amino acids except lysine. But the leucine is not different from other amino acids as far as the N^{15} content is concerned. It seems the dextro acid is completely deaminized and part of the nitrogen has gone back to form the l-leucine.

STANLEY: There appears to be one exception to the rapid continuous synthesis and degradation of protein in living cells. Spencer's studies on the metabolism within virus diseased plants indicate that when the protein is largely in the form of virus, it is no longer rapidly degraded and appears to be unavailable for food. In other words tobacco mosaic virus protein appears to resist degradation within the plant.

RITTENBERG: There are also other proteins, such as tendon, which resist degradation. It should be interesting to test the process in the virus.

MIRSKY: When an amino acid is replaced in a protein, does the replacement occur in the intact protein or is the protein actually broken down and to some extent built up again?

RITTENBERG: That is a very difficult question and I am afraid that I cannot answer it. If there is a total breakdown of protein in order to introduce one leucine molecule, you are going to have a very rapid rate of breakdown. You must suspect that the half-life of proteins must be of the order of one half-hour or even minutes. However, if you assume that you can take something out and put it back in, you do not need such a rapid rate. It is my opinion that one half-hour is too fast. I tend to believe, although I have no direct evidence, that you can take out a piece and put it back again without disrupting the whole molecule; something holds the molecule together. Do you not think so, Dr. Wrinch?

WRINCH: Of course I think so.

CHILD: Do you think any of these processes go on in vitro?

RITTENBERG: I do not think any of these would go on in vitro. The formation of peptide bonds requires energy which is not readily available in a test tube. But degradation could occur.

CHILD: I meant, if you had a solution of egg albumin and added glycine to it, how much of it would exchange?

RITTENBERG: None.

UBER: To what extent have you been able to keep a mass spectrograph in operation?

RITTENBERG: Our mass spectrograph is a routine instrument and has been in continuous operation since we built it. We have never been in a position where we could not analyze our samples within a reasonable time after isolation.

SCHULTZ: What is the nature of the non-protein nitrogen fraction?

RITTENBERG: It is the fraction we get out of the proteins with trichloroacetic acid. We have gotten urea out of such fractions and no doubt it contains almost every amino acid in the protein but we have not been able to track it down further. We can get peptides out of it.

CHILD: Is lysine the only amino acid which holds the D/n ratio constant? Have the other essential amino acids been tried?

RITTENBERG: It is the only one we have found, but there may be others. Leucine, histadine, and phenylalanine do not. Any amino acid which cannot be substituted in the diet by its corresponding alpha-hydroxy or alpha-keto acid will probably react as lysine does.

BODIAN: Is it really necessary to assume that all areas of protoplasm are doing this all the time? If only certain active regions, such as regions of cell breakdown and mitosis, were undergoing the process investigated, a half-hour half life would not be so short. The isotopes fed in the diet may be going into just such regions.

RITTENBERG: That is a possibility. For the liver protein, the metabolically active portion must comprise at least 16 percent of the total nitrogen. I consider it more plausible that this is a general reaction. Tendon, for example, is an exception.

SCHULTZ: Might not the analogy be a little closer to the virus case if one considered the nucleoproteins? In Hevesy's work with radioactive phosphorus, he found that nucleo-proteins of higher molecular weight had a slower rate of exchange than muscle adenylic acid, for example.

RITTENBERG: There are slow proteins, such as brain protein, but in general most of the proteins

seem to participate in these reactions at a rather active rate.

CLAUDE: It would be interesting to find out if your N¹⁵ is located in certain portions of the cell.

RITTENBERG: We are now engaged in fractionating liver proteins to see if there are any differences. We have fractionated plasma proteins and found they all contain about the same concentration.

CLAUDE: But isolating proteins might not give the answer. It might be concentrated in certain organs.

RITTENBERG: This is a possibility.

STANLEY: As long as most of us are interested in nucleoproteins, I wonder if you have done any work on them.

RITTENBERG: Just in the last few months we have started to work on the purines. In pigeons the purines are very active. The rat experiments have not been completed but they tend to show that the purine metabolism is much slower. Barnes in our laboratory is doing the work but has not finished it.

STANLEY: It would look as if a simple change from the ribo- to the desoxyribo-type is an easy one to do *in vivo*; a methylation and reduction.

RITTENBERG: Sometimes the easiest chemical reactions cannot be carried out in this way. The conversion of creatinine to creatine, which is easy in the test tube, cannot be carried out by the rat.

RÉSUMÉ AND PERSPECTIVES OF THE SYMPOSIUM ON GENES AND CHROMOSOMES

H. J. MULLER

Much as we biologists rightly stand in awe of the results and methods of the physicists and chemists, it is well for us to remember that our in some respects much cruder methods have nevertheless led to fundamental knowledge which is essential for the orientation of the physicist and chemist in his investigation of the way in which living matter works. In this connection I should like to cite (with slight rearrangement) a statement from a most eminent biologist who though not with us now is in a sense well represented here.

"Claude Bernard, in 1878, . . . maintained that the cytoplasm is the seat of destructive metabolism, the nucleus the organ of constructive metabolism and organic synthesis, and insisted that the role of the nucleus in nutrition gives the key to its significance as the organ of development, regeneration, and inheritance. . . . In its physiological aspect, . . . inheritance is the recurrence, in successive generations, of like forms of metabolism; and this is effected through the transmission from generation to generation of a specific substance . . . which we have seen reason to identify with chromatin [nuclein].¹ . . . Nuclein¹ . . . may in a chemical sense be regarded as the formative center of the cell which is directly involved in the synthesis of complex organic matters. . . . [Thus] the specific character of the cytoplasm is determined by that of the nucleus. . . . The nucleus cannot operate without a cytoplasmic field [*sic!*] in which its peculiar powers may come into play; but this field is created and molded by itself."

The above passage was written by Edmund B. Wilson in 1899 or earlier, and appeared in the second edition of "The Cell" (1900). And in an article in 1911 he wrote: "Kossel makes the pregnant remark that . . . every peculiarity of the species and every occurrence affecting the individual may be indicated by special combinations of protein 'Bausteine.' The facts lead us to seek for such compounds (substances) in the chromatin or the chromosomes. It can hardly be said that even a beginning has been made in the chemical investigation of the distribution of the chromatin-substances within the nucleus. Cytologically, however, a long series of the most significant facts have been made known in respect to their groupings and modes of distribution . . . and the fact is now more than ever evident that they run parallel to the factors of determination and heredity. It is difficult to see

¹In other passages it was made clear that the words chromatin and nuclein were here used synonymously, to denote nucleoprotein, the substance of the chromosomes.

what meaning such processes can have if they do not involve a linear alignment of different substances which are thus brought into a particular disposition for ensuing processes of division (Roux) or of paired association (Strasburger)."

The present concept of the gene only carries a little further the point of view already expressed above. Only recently, however, has the long non-recognition of the gene by physiologists and biochemists in general at last given way. The gene does somehow exist, many of them now admit, even though it has chosen to make itself known through the (in physical circles) less accepted society of cytologists and geneticists, and its startling characteristics brook no further pretense of its non-existence.

The most fundamental—in fact, the unique and distinctive—characteristic of a gene is, as I pointed out in 1921 (1922, AMER. NAT. 56:32-50), the fact that, in its protoplasmic setting, it produces a copy of itself, next to itself, and that when its own pattern becomes changed, the copy it now builds is true to its new self. As genes of so many different kinds, or "patterns," exist, the genes of the present day must be very complicated; thus the uniqueness of this property of self-copying becomes all the more evident, as compared with anything known among inorganic materials. Among the constituents of cells, this property is known to exist, for certain, only in chromosomes and plastids. The reproducible changes in pattern of this gene material form the basis of evolution. Theoretically, we should be able to follow this material back in evolution to the stage where it existed alone, without protoplasm of its own construction. Such a primitive stage is today most nearly represented by phages and other viruses (Muller 1921, *ibid.*; 1926, PROC. 4TH INTERN. CONG. PLANT SCI. 1:897-921).

If this interpretation is correct, the protoplasmic constituents of cells, aside from the genes themselves, have, ultimately, been derived from the genes as a kind of useful by-product, as gene mutations occurred and were selected which led to more complex "adaptive" activities on the part of the genes and gene-products; thus the genes would, in fact, constitute the essential basis of life. I am reminded of a time when these ideas were not so acceptable, and when, in 1926, in submitting for another symposium the title "The gene as the basis of life," it was suggested that I make the slight adjustment of substituting the term "a basis" for "the basis." Today, however, I think that a large number of physiologists realize that the above point of

view has a sound scientific foundation. Recently it has received a brilliant confirmation and been made much more concrete in Stanley's work (1935 *et seq.*), which demonstrates the existence of single molecules of virus of enormous size, having these properties. Furthermore, these molecules are found to be composed of nucleoprotein, the very substance characteristic of the chromosome and plastid. Important conclusions also follow from a consideration of the kind of constituents of which this virus nucleoprotein is found to be composed (see last section).

It is evident that, in view of the above remarkable properties of the gene material and its basic, central position in life phenomena, it behooves our science of today to bend all energies possible to the coöperative study of it, both in its cytological, genetical, and chemical aspects, through an attack on chromosome structure and behavior, on phenomena of heredity and variation, on proteins in general, and, as we now see so clearly, on chromosome and virus proteins in particular. This Symposium has given the best survey of any yet attempted of work in all these fields from this point of view. There have, to be sure, necessarily been a few important omissions, especially of those concepts which center about the work of Darlington, but in these times we are fortunate that it has been found possible at all to marshall a group as large and representative as the present one for a coöperative attack in a field of fundamental science.

Our present review will admittedly be far from complete and will attempt to give details of only certain selected topics² more closely related to the central themes above referred to. Pursuant of this aim we may turn our attention first to the work on the visible structure of chromosomes under natural and experimentally controlled conditions, and to inferences that may be drawn therefrom.

THE VISIBLE STRUCTURE OF THE CHROMOSOMES AND INFERENCES THEREFROM

It is evident that cytologists as well as geneticists in general are now agreed upon the essentials of the concept of the chromonema, put forward in 1908 by Bonnevie and in 1911 by Vejdovsky. In fact they go even further—especially than the latter investigator—in attributing a genetic continuity, and even an actual material persistence through cell-cycle after cell-cycle, to this spiraling thread that constitutes the primary feature of chromosome structure. Tightly coiled in the metaphase chromosome, it may certainly become drawn out by uncoiling to ten times the length of the latter, and probably, in the case of the salivary chromosome at least, to a hundred times—unless, as seems far less likely to the present author, some kind of interstitial growth

is involved in the latter case. In the coiled stages, certainly during meiosis and probably also during certain mitotic stages, as indicated by observations of Huskins and his colleagues and of others, both major and minor spirals exist; this had already been foreshadowed in the early work of Vejdovsky.

The number of sister threads present at any given stage is still a matter of dispute and there is reason to believe that it differs in different material. Nebel has emphasized the difficulty or impossibility of deciding, by ordinary optical evidence, whether only two or four or more strands are present when (as in some of his material) the diameter of the whole structure is not more than a quarter of a micron, and when the possible existence of a minor spiral may introduce an illusory appearance of compoundness. In coccid material (Schrader, Ris, and co-workers), however, a quadripartite structure is evinced by the semi-independent movement of the different strands in anaphase, while on the other hand the evidence from the induction of gene-mutations by treatment of *Drosophila* spermatozoa strongly indicates that the chromonemas in them are single, since "fractional" mutants form only a small minority (not more than about a fifth).

Concerning the nature of the coiling of sister or homologous strands about one another, it is often evident, as Huskins has emphasized in this Symposium, that the coiling is in some sense "relational." It is to be noted that such strands nevertheless come apart readily, both in the normal mitotic and meiotic divisions and also, as Swanson remarks, when heat is applied. Hence the "relational" coiling, when extensive, must be compensated for in some way, either by being reversed at intervals as Huskins reports in his material or, as Darlington has proposed for certain cases, by a simultaneous torsion of the threads on their axes in the opposite direction. It is obvious that in ring chromosomes this compensation, of whichever kind, must be very exact, for without it there would be interlocking; moreover, if the compensatory process were more pronounced in these than in normal chromosomes we should expect crossing over and mitotic separation to be somewhat interfered with, while in fact these proceed with surprising normality in ring chromosomes. The "net" preservation of axial orientation with reference to each other, continued along the whole length of the chromonemata, which is implied by these facts, leads unavoidably to the inference (necessary also on chemical considerations) that the chromonema is not radially symmetrical in its cross-section, but is polarized in this plane; that is, that it has a differentiated "face" or faces along which it reduplicates and preferentially synapses. The fact that synapsis of the threads tends to be in twos, even when more than two identical threads are present, further indicates, in the present author's opinion, that there is probably just one pairing face and that the pairing and duplication must therefore be "face to face," not "face to back."

² Unfortunately the important paper by Rittenberg, on the results of experiments in which tagged atoms were used, came too late in the series to be included in our present review.

This in turn would imply that the gene, if not bilaterally undifferentiated or symmetrical, is at least bilaterally complementary in its pairing and duplicational properties.

The coiling of the chromonema does not necessarily take place by one type of bending of a given face. For, strange to say, the direction of the coiling does not seem to be an expression of peculiarities of the molecular structure of the nema itself, inasmuch as the direction of coiling changes randomly at the centromere and at chiasmata, as pointed out by Huskins. This fact shows the direction of coiling to be under no "unitary control," resident in its molecular structure or elsewhere. Rather is the direction of the coiling a phenomenon imposed upon the chromonema from outside itself, by forces connected with the sister or homologous strand, the "sheath," or other material. This is not to say, however, that the chromonema itself plays an entirely passive part in the whole coiling process, nor that its constitution has no relation to the general nature of the coiling. It is not unlikely, moreover, that there is coiling on a molecular and even intra-molecular scale as well as the grosser coiling, and all of this would have to be of a regular kind, not like the heterogeneous folding and crumpling found by Mark in his analysis of the stretching and contracting of rubber. It is further to be noted that, in some cases, the rungs of the spirals may form the basis of apparent chromomeres, although this can by no means be the sole explanation of all chromomere-like structures seen.

Special attention has rightly been paid to the relation of ordinary chromosomes to the Dipteran salivary gland bodies which, since the work of Painter and Heitz, are known to be chromosomal in basis, for it is important to know in what ways and to what extent these bodies may be taken as providing a magnification of normal chromosome structure. It is evident from the work of Metz that, although these are chromosomal, the earlier conceptions of them were in some important respects too simple. In the first place, they are not hollow cylinders with chromonemata lying in the periphery alone, but are substantially alike throughout their cross section. Secondly, the inferred chromonemata within them must be conceived as invisibly fine, and not identifiable with the strands cytologically observable in them. This is shown, among other things, by the fact, noted also by Painter, that the number of apparent strands varies from one region to another along the length of the chromosome. These visible lines are even seen to cross each other sometimes, which could not be the case if they were actual chromonemata, lying in the same plane, as some of these do. Moreover, some of the lines are plainly seen to result from stretching, and lie in the direction of the pull. The appearance of droplets, often observable in the chromatin-poor regions, would allow of the existence of a continuous phase between the droplets which was capable of deformation or

rearrangement in various ways, and so of giving the appearance of lines in one or another direction. And even the chromatin itself shows varying degrees of dispersal or, as Painter might term it, of bunching. Finally, the chromosome as a whole may shrink and swell in its cross-sectional thickness, often reversibly, in response to the application of salt solutions and under other abnormal as well as under apparently normal physiological conditions.

While the above complications are not brought forward in actual disproof of the compound or "polytene" character of the salivary chromosomes, they do show that the conspicuous features of the visible appearance of the mature salivary chromosomes are not to be interpreted simply in these terms. In their earlier development, however, the situation is simpler, as might have been expected, and here we do find more direct evidence for the polytene conception. As Painter has shown, the chromosomes in the earliest stages of the salivary glands can be seen to double and redouble, i.e., to increase by powers of two, until the still increasing groups of threads become too obscure and compound internally for the individual strands to remain identifiable. Even to the end, however (as indicated in some of Bridges' figures, for instance) vestiges often persist of the earlier divisions, traceable as a tendency for the chromosomes to appear as though compared not merely of two (the homologous) strands, but for these two in turn to appear composed of two lesser ones, which again may show further internal dichotomy. This sort of evidence, taken together with the studies of earlier investigators which show a polymodality of nuclear volumes—indicating their discontinuous growth by a doubling process—give strong support to the conception that in the growth of the salivary chromosomes there has been a repeated multiplication of parts rather than a mere increase in the size of the original. If however we use the amount of increase in nuclear volume, or number of volume modes, as an indication of the number of doublings that has occurred, we find that the finished chromosome pair contains over a thousand strands (indicative of nine doublings), at the least. Considering the comparatively small diameter of the salivary chromosome, this would make the individual strands too small and close together ($< .1\mu$) to be resolvable one from the other by means of visible light. Thus the polytene view itself, logically followed, leads to the same conclusion as the other evidence: namely, that the visible threads cannot be actual chromonemas.

If in addition we assume that the chromonemas in the final chromosome remain, individually, no larger than in the mitotic chromosome, and use the volume of the latter as representing the maximum volume of the former, we find that, in order to have the great length seen in the salivary chromosome, they can at this stage have diameters no greater than about 200 Å. This would make them not merely

quite invisible, aside from accessory accretions of material, but even of a smaller order of magnitude than that calculated by the method above given. The difference would have to represent some kind of accessory or inter-chromosomal material—sometimes called “matrix”—which on this view would have to constitute by far the greater part of the volume of the salivary chromosome.

Another important question concerning the salivary chromosomes concerns itself with the individuality of the chromomeres or discs. As Painter's and other work shows, these increase greatly in apparent number during development. There seems at present no sure means of knowing that they have attained their ultimate possible dissociation in the mature gland cells. It would be hazardous to assume that in this stage they necessarily represent individual genes, both for this reason and because work on the genetic subdivisibility of limited regions shows that the genes, even giving them their maximal possible dimensions, are at just about the limit of optical resolvability.

The answers to the various questions raised above would be seriously affected if the salivary chromosomes were only modifications of the “lampbrush” type, as their appearance after treatment with alkalis has been taken to indicate (Calvin, Kodani and Goldschmidt, 1940). Attacking this problem, Painter has brought forward evidence to show that the structures seen under these conditions could not be taken as direct indications of the normal salivary chromosome structure, but represent various disintegration phenomena, involving disorientation, solution and reprecipitation of parts. In these changes the reactions of the “matrix” appear to play a prominent part.

A different kind of chemical attack upon the structure of salivary chromosomes was reported by Mazia. He found that the visible continuity of the structure depends upon a protein that is digested by trypsin and by a protaminase, but not by pepsin, and he concludes that the substance (or one of the substances) necessary for binding the chromosome into a permanent structure is a protamine. Protamine molecules may, he believes, be large and complicated enough to constitute the genes. At the same time, it may be noted here that the protein forming the bulk of the sperm of different species of fish may vary considerably in its place in the ordinary protein classification, from different protamines to histones, and that the protein of tobacco-mosaic virus does not fall into the protamine category. Thus it still seems too early to infer that genes are necessarily of protamine composition, or that the bulk of the material binding the salivary chromosomes together is genic.

As for the nucleic acid of the salivary chromosomes, it appears to be bound to the permanent structural protein through its phosphate group, since it was found by Mazia to be removed by phosphatase, without disintegration of the visible

structure. This result indicates at the same time that the nucleic acid here is probably not much polymerized, since phosphatase is not known to work on polymerized nucleic acid.³ The isotropicity of the salivary chromosomes (first reported by Altenburg) is regarded by Schultz as further evidence for the view that the nucleic acid in them is not highly polymerized, although on stretching the chromosomes do become somewhat anisotropic.

That the “basic staining” of the discs is really due to their contained nucleic acid, of thymus (desoxyribose) type, was shown in Caspersson and Schultz's observations that they gave both the Feulgen reaction and the characteristic ultraviolet absorption curve of this substance. The ultraviolet curve also showed the maximum indicative of tryptophane (as in protamines and many higher proteins, but not in histone). Quantitative ultraviolet absorption determinations showed less than one percent of nucleic acid to be between the discs. Another portion of the nucleus which was proved by its ultraviolet absorption to contain a high concentration of nucleic acid was the nucleolus. It may be recalled that the nucleoli had been shown by Kaufmann to be products of specific loci or regions of the heterochromatin. The nucleic acid in the nucleoli is however of the yeast (ribose) type, as shown by its lack of Feulgen staining. Besides the nucleic acid, there is in the nucleoli a protein component staining with fast green, possibly a globulin or histone. That this is attached to the structural constituent of the nucleoli through the nucleic acid is indicated by Schultz's finding that ribonuclease, in disintegrating the nucleic acid, releases this fast-green staining material.

In the cytoplasm also, as the ultraviolet absorption and lack of Feulgen staining show, there is ribonucleic acid, and this was found, in certain cases at least, to be in higher concentration near the nuclear membrane. Schultz postulates that this cytoplasmic ribonucleic acid is derived from that of the nucleus, and, more specifically, from the nucleolus. It was shown by Caspersson that in certain egg stages in which synthesis is especially active there is more of the cytoplasmic ribonucleic acid, and Caspersson and Schultz believe it probable that this correlation is more than casual, i.e., that this substance, in its protein combination, plays an important role in protein synthesis in the cytoplasm, related perhaps to the role played in synthesis in general by the nucleus.

It should be noted on the other hand that Claude, while not denying the possible role of cytoplasmic nucleoprotein in synthesis, reported that chemical analyses of cytoplasmic granules which he believes to be of mitochondrial nature showed these to be

³ In studies on *Arbacia* eggs, however, Mazia obtained evidence that polymerase was there carried primarily by the nucleus (unlike the findings of Greenstein on other material, to be referred to later), while on the other hand phosphatase occurred in the cytoplasm, independently of the amount of nuclear material.

rich in nucleoprotein. He is inclined to view these granules and their contained nucleic acid as autogenous rather than derived from the nucleus.

Schultz has connected his ideas on the above topics with the phenomena of "eversporting displacements." These are the type of cases, reported by the present author, in which a gene or genes transferred by chromosome rearrangement from a euchromatic region to the neighborhood of a heterochromatic one show a position effect whose degree of expression varies somatically in a rather irregular way from one cell or group of cells to another, thus giving "variegated" or mosaic types, and even varies from one individual as a whole to another. In such cases the euchromatic region in question, now placed in the vicinity of a heterochromatic region, shows in salivary gland material an appearance approximating that of heterochromatin itself. It has been observed by various investigators (especially by Schultz and by Prokofyeva) that in groups of flies in which the phenotypic position effect is less marked the chromosome region containing the affected genes also shows less transformation, when studied in salivary chromosomes; i.e., it appears more as it did when it was in its original position in the euchromatin and shows less of the characteristics of heterochromatin. Among these characteristics are peculiarities in the concentration and distribution of the highly refracting material, that is, the nucleic acid; more specifically, according to Caspersson and Schultz, there is a higher concentration of nucleic acid. Schultz accordingly proposes that the phenotypic position effect exerted by the heterochromatin results from a higher concentration of nucleic acid in the neighborhood of the transferred euchromatic region. He postulates that the latter condition interferes with the synthetic activities of the genes in this euchromatic region, perhaps by depriving them of their normal supply of nucleic acid. In extreme cases, he believes, even the reproduction of the affected genes may be prevented, and he regards the faintness, grading into invisibility or apparent absence, of the chromatic discs in question, as noted in some of the cells, to be brought about by this mechanism. He interprets in the same way the lack of growth and apparent lack of reduplication which he has noted for Y chromosomes in nurse cells in which the euchromatic regions, in contrast, are increasing and multiplying markedly.

It must be noted that in Cole and Sutton's work, on the contrary, no disturbances in banding were observed in euchromatic regions transferred to the neighborhood of heterochromatin. In the latter cases, however, the bands studied were not as close as they might have been to the heterochromatin, and it is doubtful whether cells were selected in which the heterochromaticity of the region in question was at its maximum.

Certainly the effect of heterochromatic regions in causing regions of euchromatin that have been transferred to their neighborhood to partake to

some extent of their own characteristics seems to have been sufficiently established by various workers (including both Prokofyeva, Muller and their co-workers, and Schultz and Caspersson). But just because of the fact that the characteristics proper to the heterochromatin, and imposed by it upon originally euchromatic regions near it, are so manifold, we must be very cautious before deciding that a particular one of these peculiarities lies at the root of the observed phenomena. Among these distinguishing characteristics of heterochromatin are included not only a different concentration of nucleic acid but also a different type of distribution of the latter, peculiarities in the appearance of the "discs" or "chromomeres" and in the whole internal structure of that region of the chromosome, a marked tendency of the chromosome region to non-specific conjugation, and an inordinately high breakability, both of the simple and compound types. There is however, much opportunity for further work along these lines, and as a working hypothesis the interpretation proposed by Schultz has some very attractive features, especially in that they do permit of further testing.

CHROMOSOME AND GENE MUTATION

Turning now to studies of the manner of occurrence of permanent changes in the constitution of chromosomes and genes, we may consider first the work dealing with changes gross enough to be analyzable morphologically. It was shown by McClintock in a series of striking and ingenious investigations that mechanical breakage of chromosomes leaves broken ends that can undergo fusion with one another. If the ends that fuse are not those that were originally together, undergoing restitution, structurally changed chromosomes result. Mechanical breakages resulted in her experiments 1) from the separation of the centromeres of dicentric rings (themselves formed by an earlier breakage and refusion involving monocentric rings), 2) from the similar breakage of dicentric chromosomes formed by crossing over within heterozygous inversions, and 3) from the snapping of chiasmata whose terminalization had been impeded by the presence of a nucleolus or merely by sticking of the threads. It was found, however, that broken ends retain their capacity for fusion for an indefinite time, until given the opportunity to fuse with other broken ends, only in gametophyte and in endosperm tissue; in sporophyte tissue a regulatory process of a different kind ensues, whereby the broken ends undergo healing, to form permanent free ends. Similar mechanically induced breakages, followed by fusion of broken ends with resultant rearrangements of parts, were found by Beadle to arise in large numbers in his line of maize in which, owing to the presence of the gene "sticky," the chromosomes show exceptional stickiness and so become stretched to the breaking point. (It is important to note in passing that large numbers of "point mutations" also arise in this line.) In

Rhoades's telocentric chromosomes somewhat similar phenomena of breakage and fusion were observed. Observations of the above kinds, since they do not introduce any agent the effects of which might be manifold or debatable, do much to make clear what the inherent properties of the chromosomes are which give them the potentiality of undergoing structural change. If these properties could be boiled down into one general characterization, we might say that the origination of structurally changed chromosomes depends upon the fusability of broken ends.

Despite the doubt that so long shrouded the mechanism of its operation, the action of X-rays and similar radiation in causing structural changes of the chromosomes has also become clear in recent years, at least in its main outlines, and the conclusions derived from this study, although arrived at largely independently of those referred to above, are completely in harmony with them. The X-ray work of Sax on *Tradescantia* microspores and other material, of Carlson on grasshopper spermatogenesis, of Demerec, Kaufmann, Bauer and their collaborators on salivary chromosomes of *Drosophila*, and of myself and my co-workers using genetic methods in *Drosophila*, as well as work of Fabergé, Catcheside, Dempster and various others not at this Symposium, all agree in showing that, as had early been maintained by Stadler and by Levitsky, the structural changes are caused by breakage of the chromatin at two or more places, followed by two-by-two fusion of the ends derived from different breaks. It turns out that in the origination of gross rearrangements the individual breaks, like gene mutations, are induced by individual ionizations (or excitations), but since each finished rearrangement requires two or more breaks, the ends derived from which must have united with each other, two or more ionizations are necessary for every rearrangement. Moreover, these ionizations must have succeeded one another within so short a period of time, or under such conditions, that restitution or healing of the ends derived from one break did not occur before those from the next break became available.

In spermatozoa of *Drosophila* the above period turns out to be the entire life of the sperm, until fertilization, for breaks accumulate accurately throughout their history. But in the plant material tested and probably in the resting stages of cells in general the period is measured in hours or minutes, being shorter at higher temperatures in correspondence with the greater speed of cellular processes. In accordance with this, when the treatment is given within the critical period appropriate for the case, the frequency of induced rearrangements varies as the square of the dose so long as the latter remains so small that the proportion of rearrangements involving more than two breaks is negligible. At doses higher and higher than this, the occurrence of more and more multiple-break cases causes the fre-

quency of rearrangements produced to rise less and less rapidly than the square of the dose, since more breaks become used up in the individual cases (a type of phenomenon referred to as the "saturation effect"). When only viable rearrangements, instead of all rearrangements produced, can be counted this falling off of the second derivative at higher doses is intensified by reason of the fact that with more breaks there is relatively less chance for the rearranged chromosomes to be so constituted as to be viable (monocentric). Contrasting with these results, when the time of treatment, even at the lowest doses, is of the order of the critical period and the dosage is increased only by increasing this time, the frequencies are, as expected, found to show a nearly linear relation to the dosage.

There is evidence that (as was to have been expected) propinquity of the broken ends at the time they are produced favors the likelihood of their union with each other, though this effect is probably much less marked when the breaks are produced in spermatozoa than in resting cells, on account of the far greater chance afforded in the former case for the broken ends to become moved apart before they are able to undergo union. Nevertheless, even after spermatozoon treatment, and when the dose is rather high, the influence of propinquity is marked enough to cause the great majority of broken ends to undergo restitutional union (Pontecorvo and Muller). Another probable expression of the propinquity effect is to be found in the finding that neutrons, which on physical grounds are especially likely to cause two or more breaks fairly near together (though not necessarily near together along a given chromosome), give rearrangements whose frequency is more nearly linearly proportional to the dose than when induced by X-rays (Sax). Here the existence of spatially preferential union must have an effect in lowering the exponent that expresses the frequency-dosage relation similar to the effect of temporally preferential union previously discussed.

Another factor which prevents the rearrangements from being strictly random is the preferential involvement in such changes of regions near centromeres and telomeres and of other heterochromatic regions (including, but to a lesser degree, the less markedly heterochromatic regions found by Prokofyeva-Belgovskaya and by Kaufmann in interstitial regions of normal chromosomes). It is not yet known whether this is due to a greater tendency to breakage or to a lesser tendency to the restitutional type of union on the part of such regions, or to both factors combined.

Even when all the above principles are taken into consideration, there still remain difficulties in accounting for the quantitative aspects of some of the data, as Fano has shown. Thus, some experiments cited by him indicate a failure of the multiple break cases to rise as rapidly as they should with rise in

dosage, in relation to double break cases, while on the other hand in experiments of Carlson the expected Poisson distribution was observed. It is not surprising, however, that in such a new subject much should still remain to be learned.

One of the matters not yet agreed upon concerns itself with whether or how often, and under what conditions, an ionization may result in a break in just one of two sister strands or in both, when the hit occurred after both had been formed, or, conversely, when the hit occurred before the reduplication of their common parent strand. It is reported that in Ris' work on coccids even four strands may—eventually at least—become broken by one hit. And in *Tradescantia*, after irradiation of stages containing, according to Sparrow, Sax and others, two distinct strands, both strands are sometimes found to have become broken in sensibly the same position. Some critics still question whether in the latter cases the strands had really been double when treated. If however we assume this to have been proved, we may interpret the result as having come about either by a "branching" or the effect of one ionization, or, less directly, by the breakage in one strand having secondarily exerted a strain on the nearby sister strand. If this effect, no matter how caused, were a frequently occurring one, it would seriously interfere with our use of "chromosome breaks" (those in which both sister chromatids were later found to be broken) as indications of the chromosome having been undivided at the time of treatment.

In the above respect, it may be that spermatozoa—of *Drosophila* at any rate,—constitute a special case. After treatment of this material, breakage and rearrangement is found in the great majority of cases (some four-fifths) to have involved both sister strands alike. And here it is highly probable that the chromonema was in fact still unreduplicated when the irradiation was applied (i.e. in the spermatozoon stage). For here gene mutations show the same characteristic, that is, the great majority involve both sister strands alike. It is hardly conceivable that, even in the cases where different alleles are possible and have similar chances of production, not only the same gene would have been affected simultaneously in both sister strands, but in precisely the same way. Hence the likeness of the breaks in the two strands in these cases is probably a result of the chromosome having been undivided at the time of treatment. This does not, however, disprove the possibility of corresponding breaks in two sister strands for the cases where both are present.

Whatever may be concluded concerning the induction by the same ionization of two identical or nearby breaks in different strands, it has become clear (both from the work of myself, Belgovsky and other co-workers and from that of Demerec, Kaufmann, Fano and other co-workers) that in *Drosophila* two nearby breaks may be induced by the

same ionization at slightly different positions in the same strand, so as to give rise to minute rearrangement. For the frequency of production of minute rearrangements puts them in a class apart from gross rearrangements, and the change in frequency of the former with change in dosage is linear, as is the case for gene mutations and single breaks and not for gross rearrangements. Such a linear relation affords critical evidence that single ionizations or excitations are the causative agent in the production of the given effect. Since then the effect can be seen to have involved two breaks, both must have resulted, more or less indirectly, from the same ionization or excitation.

These minute rearrangements may give rise to phenotypic characters like those of gene mutations. When they are inversions, they do this by position effects; when they are small enough deletions, through acting as "amorphs" or even (where repeats existed before) as "hypomorphs"; and when they are themselves small duplications through giving "hypermorphic" dosage effects, as well as position effects. Thus the question arises of to what extent apparent gene mutations may really consist of minute rearrangements, too small to be detected cytologically or through an influence on crossing over, and whether there is in fact any distinction other than an intergrading one, of size, between the two assumed categories. Since the frequency-dosage relation observed in their induction by X-rays has failed to provide a criterion for distinguishing the minute rearrangements from the gene mutations (that of both being linear), recourse was next had to a study of ultraviolet affects, inasmuch as this agent had been found not to induce gross rearrangements nearly so readily, in relation to gene mutations, as X-rays do. But although certain preliminary experiments (by the author, Mackenzie, and P. N. Bridges) have suggested a difference, the evidence is still conflicting, for other observations (Slizynski, *ex lit.*) indicate that in *Drosophila* minute rearrangements as well as gene mutations can readily be produced by ultraviolet. Hence it may be that this criterion also will be inapplicable.

Nevertheless, despite these in a sense negative results, there remain lines of evidence in *Drosophila* that argue for the conception of the gene as a definitely delimited part of the chromonema, and, as a corollary of this, for the validity of the distinction between intra- and inter-genic mutations. Among these points we may note: 1) the fact that broken ends do not behave, in uniting, as though possessed of opposite sign and hence cannot be products of breakage between peptide links but must represent separations that occurred between considerably larger groups of atoms; 2) the fact that genetic analyses of independent breaks that occurred within limited regions show the breaks often to have recurred in sensibly identical positions; 3) the fact that these breaks have not, in the cases studied, effected a complete, permanent destruction of any

of the detectable phenotypic functions of the genes or genetic material located at or near the point of breakage; 4) the fact that normal crossing over, as well as mitotically caused breakage of dicentric chromosomes, appears to occur as a result of a relatively slight (in comparison with the strength of chemical bonds) mechanical pull. While these considerations indicate that the chromosome parts which are separable by breakage are of comparatively large size, it is difficult to imagine that all "gene mutations," like the visible structural changes of chromosomes, could consist only of linear rearrangements of these parts amongst each other. For the multiplicity of kinds, and of possible kinds, of "genes" is so vast that (considering that their own size could not, at most, be so very much larger than that of the above inferred separable parts) insufficient scope for their diversity and internal complexity would seem to be afforded by such a relatively gross mechanism.

The above inference concerning the differentiation of mutations into extra- and inter-genic appears to be in harmony with the findings of Stadler on induced and spontaneous mutations in maize, although the results in this material show striking and puzzling differences from those obtained in *Drosophila*. For one thing, there appears to be no position effect exerted by cytologically observable rearrangements in maize. Hence apparent gene mutations, since they have a phenotypic expression, could not so readily be interpreted as rearrangements of essentially the same kind as the cytologically observable ones, unless they were all assumed to be minute deletions. But the latter interpretation also would encounter various difficulties, when applied to spontaneous and ultraviolet but not when applied to X-ray mutations. For in maize the X-ray mutations that at first sight appear to be gene mutations are found, in general, to cause reductions of viability, when homo- or hemi-zygous; moreover, where a gene is capable of affecting various characters in its different allelic forms, X-ray mutations are found to involve simultaneous changes, of a type suggesting losses, of all these characters at once, as though there had in fact been a small deletion. On the other hand, in the case of ultraviolet and spontaneous mutations of the same loci, the characters are usually affected singly and without the above noted viability effects. Thus there appears to be no real intergrading here between the effects due to minute rearrangements and those due to gene mutation, and, in addition, their mechanism of production (the first by X-rays and the second by ultraviolet and spontaneously) seems to serve as a criterion between them in this material (though perhaps not in the *Drosophila* material). To be sure, the latter criterion is not absolute, since not only X-rays but also ultraviolet irradiation are capable of producing some real chromosome breaks with rearrangement. But the ultraviolet does not produce nearly so many breakage re-

arrangements, in relation to the gene mutation frequency, as do the X-rays. In this connection, moreover, it is to be noted that in maize the ultraviolet, unlike the X-ray breaks, in most cases appear to include at least one healed end. Hence, it is conceivable that the breakages produced by this agent are, unlike the X-ray breaks, only secondary effects of a kind of gene mutational change, that involves the conversion of an interstitial gene into an end gene or "telomere."

Another peculiarity of the ultraviolet mutations in plants (Stadler, Swanson) is an apparent delay in completion of the mutational process until after the formation of the two sister chromatids, together with restriction of the effect, ordinarily, to one of these strands (thus causing the appearance of a "fractional mutation"), whereas the X-ray effects usually appear in the products of both strands alike (as "whole mutants"). An alternative interpretation of this difference would lie in the supposition that two chromatids already exist as separable structures at the time of treatment and that while ultraviolet affects but one of these strands X-rays more often affect both alike. There is however support from another direction for the first interpretation: that the ultraviolet mutation is not finished until after fertilization and various processes of chromosome development have intervened. This is found in the fact that many more ultraviolet mutations are to be found in the endosperm than in the sporophyte derived from a treated pollen grain of maize, despite the fact that the pronuclei destined for these two structures appear to be interchangeable at the time of treatment. In this connection, it is suggestive that "healing" of broken chromosomes occurs in the latter but not in the former tissue. This should serve as a warning against the too simple and extreme conclusion that, in such material at least, structural changes and gene mutations have nothing fundamental in common at all. For if the "finishing" of actual gene mutations in the sporophyte has been prevented by something akin to the healing of broken ends of chromosomes we should have to admit some essential similarity, even in this organism, between the process of visible structural change and that of ultraviolet gene mutation. A like inference might be drawn from the fact, previously noted, that in the "sticky" strain of maize found by Beadle not only visible structural changes but also apparent gene mutations arise "spontaneously" with unusually high frequency; these may, however, be of the deficiency type produced by X-rays.

The studies of Hollaender on ultraviolet irradiation of fungi have given clear evidence of the induction of mutations here by this agent. In his earlier work, however, a difficulty in the interpretation of the results arose from his finding that, whereas the frequency-dosage relation was essentially linear for the lower doses, the observed mutation frequency at higher doses not merely did not

rise so rapidly but actually fell off with increased dose. Hollaender has now succeeded in showing that at least a part of this falling off depends upon the fact that, at the higher doses, there is a lesser capacity for germination on the part of the mutated spores, as compared with the non-mutated ones. For, when the conditions for germination are improved by certain treatments, following irradiation, the linear portion of the frequency-dosage curve is distinctly extended.

The type of dependence of the mutation frequency upon the wave length of the rays found by Hollaender is of especial interest. Not only does the curve show the same peak as that shown by the ultraviolet absorption of nucleic acid (somewhat as found in maize by Stadler and his co-workers), but in addition it shows a secondary peak, at shorter wave lengths, as expected for the protein absorption. This result would lead to the conclusion that ultraviolet absorption by either component of the nucleoprotein is capable of leading to mutation, and that accordingly, in one or the other of these cases at least, there is a transfer of the energy, or a chain of reactions, starting at the absorbing group, and secondarily reaching the material in which the final mutational change occurs.

Besides these mutational effects on the genetic material, there are also inactivational effects of another kind or kinds in fungi, that do not result in mutation. For at higher wave lengths there are more inactivated spores, for a given frequency of mutated spores among the viable ones. It is to be observed that this makes the lower wave lengths, of 3000 or, still better, 3100 Å, more efficient in practice (i.e., when the dose is stepped up towards the tolerance limit) for the production of mutants, even though, for a given amount of energy absorption by the living material (and still more, for a given energy output at that wave length by the source of irradiation) a lower frequency of mutations is induced by the longer waves.

Comparing the frequency of given mutations, presumably involving individual genes, in widely different types of organisms—viruses, bacteria, and *Drosophila*—Gowen found that, for a given dose of X-rays, there was a strikingly similar quantitative result, inasmuch as the differences found were little greater than one order of magnitude ($10\times$) in the most divergent cases. On the other hand, the rate of apparent inactivation, as distinguished from mutation, of the genes, showed no significant agreement. This latter result was not surprising, owing to the diversity of the mechanisms whereby "inactivation" may be produced, especially in higher organisms, and the difficulty of distinguishing the different types from one another. It may however be observed, in passing, that the preliminary evidence from other studies of induced mutation in *Drosophila* indicates that in this form mutations of genes as well as breakages in their close neighborhood, not involving their loss (or, at any rate,

not their complete loss), seem to occur oftener, at least in euchromatic regions, than individual losses of the same genes. This, if substantiated, would disagree markedly with the virus results, in which the frequency of inactivation—here equivalent to loss—considerably exceeds that of mutations which leave the virus able to undergo duplication.

The X-ray inactivation of viruses was found by Gowen to be of approximately the frequency expected on the "sensitive volume" assumption, according to which practically every ionization produced within the virus, and none produced elsewhere, results in its permanent change: in this case, inactivation. Differences were reported, however, between the effectiveness of different wave lengths of X-rays, that would be difficult to reconcile with a strict application of the sensitive volume interpretation. While such differences were conspicuous by their absence in the similar experiments of Luria, reported in the discussion, it may here be noted that in work shortly afterwards published by Luria and Exner (PROC. NAT. ACAD. SCI. 27:370-375) differences were noted in the inactivating effect of radiation according to the type of medium surrounding the viruses. Thus, while still supporting the "sensitive volume" idea for given cases, they are led to postulate that in some media there is another mechanism of inactivation, besides that postulated on the usual "sensitive volume" hypothesis. There does not seem to be evidence at present to show that the genes in the chromosomes of higher forms are under conditions similar to the ones prevailing in those virus cultures which give results in harmony with the sensitive volume mechanism, rather than under conditions which disturb the clean-cut working out of this mechanism.

In so far as they form the source of the mutant genes occurring in natural populations and so serve as the building blocks of evolution, spontaneous mutations are of even greater interest than those induced by artificial means. A comprehensive survey of work on spontaneous mutations in *Drosophila* was given by Plough. It was shown, for one thing, that there is a tremendous variation in the spontaneous mutation frequency between different stocks. That similar differences, of genetic origin at least in part, also characterize individuals within the same general stock is not only a practically necessary inference from this but may also be deduced from the fact, found by Plough and his co-workers, that in various experiments with a given stock the mutation rate changed in the course of the breeding. In the experiments reported the rate dropped markedly within a few generations, as though by a selection of the genetically more stable individuals. Another major fact brought out concerning spontaneous mutations is that, when other conditions are held as constant as possible, the gene mutation rate varies with temperature, so long as this is kept within the range normal to the organism, in accordance with the van t'Hoff prin-

ciple for chemical reactions. This indicates that—as has likewise been found for X-rays—an individual atomic excitation induces an individual gene mutation. Thirdly, however, the effect of “temperature shocks” (sudden drastic changes in temperature, usually to a temperature which the individual cannot long tolerate) falls outside the above category and leads to a more considerable change (increase) of the mutation rate. This result may be regarded as due to the special chemical conditions brought about by temperatures to which the organism is not adequately adjusted. It is remarkable that in these experiments such shock effects were found only in the stocks having a low natural mutation rate, and the question was raised whether the mutations so produced are of a different kind, perhaps more nearly related to chromosomal structural changes than the usual gene mutations are. However this may be, the frequency of gross chromosome changes was found to remain very low in the absence of irradiation, even when temperature shocks were applied.

As for the induction of mutations in *Drosophila* by given artificial chemical treatments or known physiological conditions, no authentic cases of drastic effects of this kind have yet been reported despite the trial of a considerable number of agents in doses approaching the tolerance limit. And even those less marked mutational effects of chemicals (comparable with the normal temperature effects) for which fairly good evidence has seemed to have been presented from time to time by scattered workers have not as yet been followed up and confirmed by others. Admittedly, however, this field deserves to be worked much more, for there seems no sufficient theoretical reason why such effects should not be found. The greatest obstacle to the study of this matter lies in the fact that a combination of very large-scale work with high genetic accuracy is necessary in the prosecution of it.

One reason for the inference that chemical treatments may be found to influence the mutation process lies in the results which have been obtained in studies on particular genes of inordinately high potential mutability. It was proved by Demerec in *Drosophila virilis*, where three loci containing such genes were studied, and by Rhoades in his experiments with the *a* gene in maize, that such genes are extremely sensitive to influences arising from other specific genes, which instigate or inhibit mutations of a definite kind in the former genes. In these cases only certain alleles of the locus in question were affected, and the mutational effect was also largely limited to particular body regions and stages of development. Sometimes too it occurred only under specific environmental conditions (for instance, at a low temperature in one strain of maize). The evidence did not indicate that any chromosomal aberration, in the ordinary sense, was involved. And in the different cases the factors conditioning the mutations were very different and ap-

parently unrelated. It is too early to know in how far the findings in such cases may be carried over to assist us in our understanding of mutation processes in genes in general. But it is tempting to think that in such cases one gene may be poisoning or, as it were, decoying another by providing products which the latter “mistakes” for its own proper supply of substrate metabolic material—as, according to one interpretation, sulphonamides substitute for para-aminobenzoic acid. As yet, however, such suggestions remain fantasies, for students of genes in higher organisms still lack means for the detailed biochemical analysis of the ultimate objects of their study.

ATTACKS MADE PRIMARILY THROUGH BIOCHEMISTRY AND PHYSICS

The transition from the more strictly empirical or biological to the more strictly analytical and physical was provided by the epochal studies of Stanley on viruses, some of which may, as previously mentioned, be classified as naked genes. It is probably only a coincidence, however, that the tobacco mosaic virus, which was found to have a molecular weight of about 48,000,000, is of approximately the same size as that which has been calculated as a maximal estimate for the size of a gene in *Drosophila*. For the smallest known viruses, such as, for instance, a phage of staphylococcus, have a molecular weight somewhat less than one hundredth of the above size. Chemical analysis of the tobacco mosaic virus gives, among other results, the following very significant findings: 1) this substance is a nucleoprotein; 2) its nucleic acid is of the ribose (yeast) type, not polymerized, and only loosely bound with the protein components (some being separable by pressure); 3) the protein is formed of the ordinary amino-acids, without that excess of arginine which characterizes the protamines.

From the above results it is legitimate to conclude that the special peculiarities of thymus nucleic acid and of protamine—despite these being the chief constituents of some types of chromatin—play no part in the explanation of the most distinctive chemical activity engaged in by the gene: that of “self-copying” or “autosynthesis” of whatever pattern (“mutant” or “normal”) happens to be present to begin with. Nevertheless it is possible that in the type of chromatin which has been regarded as the more usual the genes may be composed of the last mentioned kinds of nucleic acid and protein as physiological or mutational alternatives to the substances found in the normal virus (see first paragraph below). But, whether or not thymus nucleic acid and protamine exist in the actual genes or in accessory substances (which would in such cases constitute the bulk of the chromosome material) their special properties must have some function or functions other than those involved in the primary process of autosynthesis.

For the present evidence shows that the latter functions are adequately subserved by ribonucleic acid and by protein constituted of amino-acids in their more usual proportions.

Divers mutations of the tobacco mosaic virus have been observed in the laboratory, and in addition variant strains, presumably connected with the standard by one or several mutational steps, have been found in nature. Among the latter are types having distinctly different proportions of certain amino acids, and different serological reactions from the standard strain. When however the composition of the virus molecule was artificially changed, as by the attachment of acetyl groups to many of the amino acids or by the alteration of the tryptophane by application of formaldehyde, the remodelled virus, while still able to reproduce, gave rise to daughter molecules of the original, standard type. That is, the above changes had not involved either the features of structure necessary for autosynthesis itself, or those which distinguish one mutant type from another one; they may rather be compared with those impermanent changes which must take place in a gene during its life history and functioning but which leave no lasting impress upon it. Further direct chemical studies along these lines will be awaited eagerly by those interested in the nature of the gene.

The picture of tobacco mosaic virus which had already been arrived at by Stanley through his studies of its chemical composition, diffusion and sedimentation rates, etc., and by Bernal and Fankuchen (see below) through X-ray diffraction analyses, has received direct confirmation through the observations and photographs recently made by Stanley with the electron microscope. The particles were seen to have the dimensions $2800 \times 150 \times 150 \text{ \AA}$, as had been calculated, and they were seen often to be rather precisely and characteristically oriented with respect to each other. No particles in an intermediate stage of development were found, although considerably smaller particles would certainly have been visible. This is illustrated by the fact that, when the virus particles had been exposed to solutions containing their specific antibodies, the "fuzziness" of virus outlines now gave evidence of the existence of the latter molecules as much tinier rod-shaped bodies, clinging to the surface of the virus somewhat like leeches—perpendicularly when crowded and more or less tangentially when given more room by dilution.

The above are the most illuminating of the results so far reported on biological material by means of the electron microscope. Zworykin's account of this instrument as made by the Radio Corporation of America in its present day form—an achievement for which he deserves much of the credit—aroused high hopes.* One wonders whether the

introduction of this instrument into biological research will result in the ushering in of a new era in biology somewhat as happened when, shortly after the invention of the oil-immersion lens and Abbé condenser, mitosis and the chromosomes were discovered, and the foundations of the chromosome theory of heredity were promulgated. For the electron microscope achieves an increase of magnification considerably greater than those inventions did, and it is very probable that in the range of magnitudes thus opened to our view structural elements of high biological importance exist.

There are, however, very serious difficulties or limitations still confronting the application of this instrument to biological material. For the present generation, much will depend upon how far and how soon these difficulties can be overcome, so that it is highly important for us to recognize them clearly. Unfortunately they are to some extent simply the other side of the very virtues of the instrument. Among them may be listed: 1) the fact that, since the structures seen are so very small ordinary cells and other objects of the size commonly used for observation under compound microscopes are far too large, so that parts of them must be isolated, at the risk of their injury or destruction; 2) the fact that there are no appreciable refraction images, but only shadows caused by density differences, on the one hand allows the picture to be less confused but on the other hand limits the type of structure which can be seen and the extent to which it can be seen and sets a premium on the observation of objects in the dry state, surrounded only by the vacuum required within the body of the instrument; under such conditions most biological material dies or becomes abnormal, and too condensed for due discrimination between its parts; 3) the small size of the objects seen and the great magnification of them and of their movements brings us into a domain where—in living matter under the usual conditions of life—the Brownian motion is so marked as to offer great hindrances to observation.

As in the attainment of the best results with oil-immersion lenses much depended upon the development of appropriate methods of fixing, staining and sectioning, so here it may be that new principles must be worked out to govern the preparation and selection of material for the electron microscope. It may be, for example, that the salivary chromosomes which, by reason of their size, furnish the best chromosomal material for study with visible light, are *ipso facto* least well suited for the electron beam, and that for this the most tenuous individual strands of ordinary resting nuclei would be most suitable. Further, for the freeing of the given components of these and other objects from extraneous material, digestion by one or another enzyme as well as treatment with various solvents might be

*For an earlier account of the history and structure of this type of magnifying device, including the important inventions of Knoll and Ruske, Martin and others, the reader may

be referred to the article by Martin, in *NATURE*, 1938, 142: 1062-1065.

of considerable help. Where the objects to be studied are of sufficiently high density, they might be kept in a more normal and expanded condition by allowing them to remain in the wet condition, in a very thin layer, protected from drying by a relatively impervious membrane, and/or by having them in a frozen state during observation. To some extent this has already been tried. Such freezing should, however, if we may judge by Luyet's experiments, be carried through very rapidly and to a very low temperature, so as to avoid the formation of ice crystals and to allow the attainment of that "vitreous" state in which the normal relations of the particles as they existed in the original liquid are largely maintained. This extreme frozen state would at the same time minimize both Brownian movement and the sublimation of the water, and also reduce the speed of any degenerative processes occurring in excised material. To counteract the warming effect of the electron beam on the frozen specimen, the wire mesh holding it might have to be made still finer, and to be modified in additional ways for conducting off the absorbed heat. Where objects of a density approximating that of water itself, or lower, were being dealt with, the water would have to be replaced by material of a lower density, such as an alcohol or hydrocarbon, likewise kept at the very low temperature.

It is evident that all this calls for the mastery of a considerable body of technique, at present not in general use. In the development of this much patience as well as ingenuity will be required. Further adaptations of the magnifying instrument itself to the above needs of biological work are also to be hoped for. In the meantime, work with the less exacting and simpler electron microscopes of relatively low magnification (about $5000\times$) that have recently been patented by the General Electric Company may afford a readier insight into the fields of view lying more immediately beyond that of our ordinary microscope, and so give useful practice in the general direction in which we wish to move.

The electron microscope does not—as yet at least—appear to offer opportunity for learning much about the inner constitution of genes or other protein molecules. Thus, beyond the present reach of even this instrument and before the orderly domain of classical chemistry is attained, there still lies a wide no-man's-land in which until yesterday definite and secure knowledge could be obtained only in occasional snatches, at the cost of a difficult and uncertain struggle and in the absence of guiding principles for the attack. But at last there are signs of somewhat more definite progress even here. This was especially evident in the enlightening and delightful exposition given by Fankuchen of the exploration of parts of this region by the X-ray diffraction method. In listening to this many of us experienced a kind of revelation which, unfortunately, we realized we might never be able to visualize again!

Giving the results of the application of this method by Bernal and himself to the tobacco mosaic virus, Fankuchen pointed out that the dimensions of the particles, or crystals, turns out to be approximately 3000 or 1500 (the length being the least well determined dimension) $\times 150 \times 150$ Å, a conclusion in substantial agreement with the figures obtained by Stanley by other methods, as previously mentioned. These particles give evidence of being strongly oriented *inter se*, when they are in a comparatively concentrated suspension and hence close together. With the addition of water, their distances apart become much greater, evidently because of electrostatic repulsion. Even when over 1000 Å (0.1 μ) apart, however, they still appear fairly regularly spaced. On the addition of NaCl to the water, they move in closer together, doubtless because of the loss of some of their charge, but a minimum of three to four layers of water molecules is found always to remain around the virus crystals in such cases.

Using larger angle X-ray beams, which would be indicative of finer structures, still more definite patterns are observed. These show the existence of a very constant arrangement inside of the virus crystal. This, unlike the arrangement of whole crystals *inter se*, proves to be little affected by the presence or absence of water or by other differences in conditions. This inner arrangement involves the presence of regular repeat-units having dimensions of about $68 \times 88 \times 88$ Å, and, within these in turn, sub-units of approximately $20 \times 22 \times 22$ Å. Evidence is also to be found of still smaller subdivisions, within the latter, of some 11 Å in each direction; these perhaps correspond to small groups of amino acids but, as is to be expected, they are not so uniform as the units of higher rank. It is evident that we now have a sort of scaffolding to which any more detailed theory of the structure of the virus protein must fit. And although we still remain largely in the dark concerning the chemical interpretation of the repeat units and sub-units of the two higher ranks, the ascertainment of their existence, size and arrangement marks a new triumph of analysis by this remarkable system of physical and mathematical techniques. This achievement goes far to refute the claims that have at times been made that the physical method which lies at the basis of it cannot be successfully adapted to the study of highly complex organic compounds.

A creative picture of the geometrical and chemical details of inner structure, as well as of the intermolecular groupings, which "native" protein molecules in general, including those of viruses, may have has been presented by Wrinch. While it would be an impertinence on the part of the present reviewer to take sides between such masters in this field as Langmuir and Wrinch on the one hand and Bernal and Bragg on the other, it must be confessed that there is much which must appeal to the biologist in the exposition which she has given

us of the morphology and properties of the particles of globular protein. And certainly, as she points out, the conclusions reached by Crowfoot and Bernal on the basis of their X-ray diffraction studies of the insulin molecule do agree with her more general thesis that there is a strict and regular three-dimensional pattern or "fabric" of parts in such proteins. Furthermore, we may note that, whatever the decision may be concerning the cyclol bond, the very definite results reported by Mirsky on the differences in availability of the SH radicles of egg albumen for entering into test reactions under different conditions confirm the conception that in a native protein the "R" groups may be covered in consequence of the folding of the molecule. This at least argues against the earlier conception of the native protein as consisting only of an extended, naked chain, held together simply by its peptide links. And as for the peptide links themselves, the lack of digestibility of many native proteins—also mentioned by Mirsky—indicates that even these links have undergone some alteration, which in their case can hardly have been caused by their being themselves folded in, inasmuch as many if not all of them would be expected to remain on the outside of the folded molecule: for the change in them, the cyclol bond would appear to offer one possible interpretation.

The above mentioned experiments of Mirsky with egg albumen indicated the existence of some all-or-none reaction in the unfolding of the molecules, in that they behaved here either as completely native or as completely denatured protein. In the denatured state, the results from his and Rothen's sedimentation and diffusion tests led them to the conclusion that the molecules had in fact opened up fully, so as to form the long chains (here about 100 times as long as broad!) postulated on earlier theories of protein structure in general. On removal of the urea which caused the denaturation, there was a partial refolding, as indicated by the SH reaction and other tests, but the return to the native state was proved to be imperfect in this case. In other cases however—e.g. in trypsin and in pepsinogen (though not in pepsin!)—the denaturation reaction was found to be perfectly reversible, with a definite equilibrium between the opposite reactions and an amount of entropy difference between the opposite states that was measurable through the effect of temperature. The unfolded (denatured) state was the state of higher entropy, as is to be expected in a case like this in which the folding is definite while the unfolding, even if chemically complete, gives irregularly extended and disoriented molecules. This result contrasts with the cases of rubber and nylon analyzed by Mark, in which the folded state, exhibiting the heterogeneity and disorientation of the thermal agitation which causes it, necessarily has a higher entropy than the unfolded state, in which a marked degree of orientation has been brought about by the

mechanical stretching process. In the cases of perfect reversibility of protein denaturation, the question arises whether the unfolding process may not be less complete than in egg albumen, for it would seem plausible to postulate that some connections besides the peptide links have remained here, to guide the refolding back into the exact pattern which it had before. Very small differences in the nature of the protein, and in accompanying substances and conditions, were found sometimes to determine whether or not the denaturation was of such a nature as to be completely reversible.

It is to be hoped that the elegant chemical methods of attack here opened up will soon be used on an increasing scale, as they appear capable of yielding critical evidence on some of the most mooted problems concerning the proteins. Among other bearings which such work may have, we may note here, as more especially interesting for geneticists, the possibility, indicated by the results on the reversibility of folding, that (as was once suggested by Wrinch) the genes themselves might, at least at the time of their reproduction, have the more or less open structure considered more probable for them by geneticists, while at the same time retaining for themselves or their products the capability of changing into more typically globular proteins for the carrying on of other activities.

In the work reported by Rothen, proof was given that the biological activity of a protein—in this case, insulin—may be retained even after it has been denatured by being spread out in a monolayer having only the thickness of a polypeptide chain, although in the case of certain other protein hormones tested the characteristic effect on the organism did disappear with such denaturation. This will serve as a warning that (as is to be expected) not all the special biological properties of a protein depend upon the retention of the precise structure of the molecular fabric as a whole. At present we do not know what features of the insulin molecule are more especially responsible for its biological effect, nor whether, for example, certain fragments of it would be active by themselves. Moreover, as Wrinch pointed out, it is not necessary to assume that the insulin molecule has opened out completely into a simple polypeptide chain in the monolayer; in fact, it exhibits certain peculiarities suggesting that it might be more capable than most proteins of retaining essential features of its fabric even under conditions in which it had become flattened out.

It was likewise shown by Rothen that some proteins retain the potentiality of entering into their characteristic antigen-antibody reactions after they have been denatured by formation of a monolayer, of polypeptide thickness. This is sometimes the case also when it is the antibody rather than the antigen which has undergone the spreading-out process. In the case of horse globulin, however, it is observed that the serological potency is lost by changes that appear to occur spontaneously in the

monolayer when it stands a comparatively short time, unless it has been held rigid under compression. Whatever the sensitive structures that have undergone the latter change may be, it is not surprising to find that there are some features of the molecular configuration which are not essential for given serological reactions. For the fitting of antigen with antibody need not be so extensive as, for example, that which a gene must undergo with its substrate in forming another gene. In this connection we may recall the observations of Stanley, previously mentioned, which showed that the molecules of tobacco mosaic virus antibody are far smaller than the virus crystal itself and that they merely adhere detachably, either endwise or sideways, to the side of their mammoth antigen.

As the work of Greenstein served to emphasize, not only the protein, but also the nucleic acid component of chromosomes—at least, that of desoxyribose (thymus) type—is capable of forming huge molecules. He was able to measure the amount of polymerization involved here through studies of the birefringence, the viscosity, and more especially the structural viscosity, of nucleic acid solutions. The last mentioned property is measured by the amount of change in resistance to flow on application of pressure, and is caused by the molecules becoming oriented in response to the pressure; the magnitude of the effect here depends on the degree of elongation of the molecules. At 2 percent concentration the thymonucleic acid was found to become polymerized to such an extent as to have (according to viscosity determinations) a molecular weight as great as 40 to 80 million. A certain amount of depolymerization was occasioned by dilution, by the addition of salts, of amino-acids, and of denatured proteins (in the latter two cases in proportion to the number of amino units present). These effects were largely additive, and they were reversible. A certain specific protein or proteins, apparently of widespread occurrence and found in various sera, milks, tumors, etc., had such a pronounced, reversible effect that it may be designated as a depolymerase (*cf.* Mazia). As it is heat-labile it appears that the existence of this protein in its native state is important for its union with the nucleic acid. Presumably, then, its denaturation followed by renaturation (as described for trypsinogen by Mirsky) would entail a parallel polymerization and depolymerization of any nucleic acid with which it was associated. While it seems too early as yet to apply these conceptions directly to such problems as those of the spiralization of chromosomes, it is apparent that they might be of fundamental significance in their solution.

Another finding along this line which is of interest to geneticists is the result obtained by Greenstein and Hollaender in an investigation of the effects of ultraviolet light upon sodium thymonucleate. Their study showed that this agent too causes a depolymerization of the nucleic acid, as

tested by structural viscosity and birefringence measurements. There was no release of ammonium or phosphate, such as would be caused by radical chemical changes, and in accordance with this the absorption spectrum for ultraviolet showed no sensible change. Nevertheless, the depolymerization proved in this case to be irreversible and was therefore of a different, more drastic type than the seemingly "physiological" depolymerization occasioned by the agents previously mentioned.

It is legitimate to classify gene nucleoproteins as enzymes, in view of the established fact of genetics that they are able to effect the synthesis of specific types of organic compounds, namely, of other molecules of gene nucleoprotein like themselves, without themselves becoming used up in the process.⁵ It is furthermore evident that, in accordance with the accepted usage of biochemists, they are to be classed in the category of protein-synthesizing enzymes. As yet, however, no distinction is known between protein hydrolyzing and synthesizing enzymes. As far back as 1906, in Loeb's "Dynamics of Living Matter," the surmise is to be found that protein synthesis and protein hydrolysis are merely opposite phases of the same enzymatic reaction, as the synthesis and hydrolysis of certain other substances were already known to be. Since then it has been abundantly proved that this relation does hold for proteins. We might accordingly be led to conclude that the gene nucleoproteins, since they are known to be synthesizing, must be identical with protein-hydrolyzing enzymes and hence should be classed as "proteases."

It is however somewhat too early for this identification because we do not yet know what level of synthesis the substrate upon which the gene acts has already attained. That is, if the building blocks which are provided for the gene as its raw material are already fairly elaborate polypeptides or proteins it may be that no further dehydrolysis is necessary and that the final steps of putting these molecules together into a pattern like that of the gene itself which guides this reaction involves the

⁵ We do not class here as necessarily enzymatic the activity of the gene in the formation of the "gene products" whereby the work of the gene in the cell is carried out. For it is conceivable that these products are derived by reactions in which gene molecules are actually used up (rather than reacting as enzymes) that have been synthesized in excess of the immediate needs for reproduction. It was this consideration which the present author had in mind when, in 1921 [*ibid.*], he stated that we should not take it for granted without more specific proof that the genes act as enzymes. At the same time, however, the autolytic property of the gene was distinctly recognized, as it had been—though without the implications based on the distinctively mutable character of the autolysis—by Troland in 1917 and by Hagedoorn in 1911 and as it had been, in a less specific way, as applied to chromosome material in general, by various earlier biologists such as Wilson, Matthews, Kossel, Weismann, Roux and others, as mentioned in the introduction.

making of some other kind of links or bonds rather than peptide links, and some other process or processes than that of hydrolysis-dehydrolysis.⁶ An example of such a synthesis by the genetic material, involving the making of attachments which though of unknown type must be of a different nature from peptide links, has in fact already been furnished through the study of structural changes in chromosomes. For it has been shown in the previous section that the connections between chromosome parts that can be separated by breakages of the kind that give rise to gross rearrangements cannot be of the peptide linkage type, being "signless." Yet in the normal synthesis of the chromosome as a whole these connections too must be copied. It is as yet unknown to what extent if at all such connections may occur within the limits ascribed to a "single gene," in the older sense (see previous section). However, the size of the gene is not so much greater than that of ordinary protein molecules as to suggest that the virtually infinite patterns of genes could all be determined merely by rearrangements of certain standard protein molecules *inter se*. As for the internal pattern of a protein molecule, it should be borne in mind that this must depend not only upon the order of peptide linkage of the amino acid units but also upon the manner of folding of the chain, previously referred to, and upon the secondary connections between the parts thus brought into juxtaposition. To what extent a given polypeptide chain has a preferential manner of folding, predetermined merely by its peptide linkage arrangement, is a matter yet to be decided, as the above mentioned studies of Mirsky and others indicate.

Despite the above uncertainties, it is obvious that a further understanding of the process of synthesis—or its converse, hydrolysis—of ordinary proteins, and of the nature of the enzymes effecting this process, may be of the highest significance in the attack on the problem of genic autocatalysis. We therefore turn with the utmost interest to the report of Fruton on the work of Bergmann, Fruton and their collaborators, for this work marks the greatest success yet achieved in distinguishing between different kinds of proteolytic enzymes and in analyzing the factors conditioning their activities. In the action of these enzymes, in general, both the formation of the peptide links, i.e., synthesis, and also their hydrolysis is induced, so that an equilibrium between the synthetic and breakdown products (with the latter in considerable preponderance) is approached, but a predominant synthesis can be maintained by continual removal of the synthetic products, by various means, from the reach of the enzymes. In the cases tested, moreover, the specificities of the enzymes were found to be alike both as synthesizers and as hydrolyzers, so that it was suf-

ficient, in studying these specificities, to deal only with the hydrolyses.

It has been found convenient to group the enzymes attacking peptide links into four main classes (designated as 1a, 1b, 2a and 2b below), according to which links they attack: (1) terminal peptide links (a) next to the "left" end (i.e., that end having a free amino group), or (b) next to the right end, and (2) non-terminal peptide links having more than one peptide link (a) to their "left" (their amino side), or (b) to their right. Classes 1a and 1b attack only polypeptides, not real proteins, thus showing that the terminal peptide groups of proteins have somehow been made unavailable, or that there are no terminal groups because of a ring or net-like configuration of the molecule. The enzymes in any one class show differences in action from each other, and from those in other classes, according to the activators (if any) which they require, their optimal pH, and the types of amino-acids, adjoining or near to the link in question, which are necessary or favorable for their action. A given enzyme is not absolutely restricted in its effect to links between just two given amino acids (e.g., to the benzoyl-arginine or benzoyl-lysine link alone), but can attack any of a larger or smaller group of linkage types; within this group, however, it usually shows preferences, attacking a given one for instance twice as readily as another one. By means of these restrictions and preferences the specificity of the enzyme is defined.

Thus far, four different intracellular proteolytic enzymes, called "cathepsins," have been distinguished. Though hard to purify, they have been found to be characterized by different restrictions and preferences as to the linkages they attack, and to have somewhat different requirements as to activators. For most of them, sulphhydryl groups can serve in activation, apparently by forming compounds with the proenzyme. As yet it is not known to what extent the constitution of these or other proteolytic enzymes must be similar to that of the substrate on which the enzyme acts, although in the case of the extracellular enzyme pepsin, for example, which is itself highly acid, an acidic group in the substrate (on the left of the link attacked) favors its activity. The cathepsins are of wide distribution, and it seems to be the opinion of workers in this field that, although some more may yet be found, the requirements of cells for proteolytic-synthetic enzymes may not call for many more.

It is observed that any given cathepsin, placed in a mixture of amino-acids, would effect several syntheses at once (though at somewhat different rates), inasmuch as it does not have an absolutely limited specificity. This would necessarily be the case so long as there were few cathepsins, to act upon a considerable number (about 23) of different kinds of amino-acids. There does not appear to be any principle involved in their action which would lead any one of them, or any combination of them, to synthesize a polypeptide or protein of just one

⁶ It is, moreover, still conceivable that the more individualized portion of a gene, whereby it differs from other genes, resides in some special portion of the nucleoprotein molecule that does not have the typical peptide structure.

specific type, rather than a mixture, still less to synthesize only a protein of just the same type as that of which they themselves are composed. That is, this mechanism by itself does not seem adequate to bring about an exact mirroring or duplication of structure, such as occurs in the synthesis of the gene nucleoprotein. It may well be that some further extensions of such work will throw light on this phenomenon, but for such a process to be effected some new principle, acting to guide the operation of the enzymes more specifically, is evidently required, or else the discovery of far more numerous and more specific enzymes.

Delbrück has given us an admitted speculation, but a very ingenious one, in the attempt partly to bridge this gap. His hypothesis is intended to show how a part of a finished protein or polypeptide molecule containing a given peptide link might become temporarily attached to and act upon the corresponding part of an unfinished combination of amino acid-like bodies of a similar type, so as to make possible in the latter the completion of the same peptide link. As the preliminary amino combinations are on this view supposed to be formed readily, numerous trial doublets of very varied types (not yet actual di- or polypeptides) would presumably be already provided in the medium, but only those would be selected out for attachment to and finishing by the original master molecule, or gene, which happened to match parts of the latter. It would have to be postulated, further, that after conjugation of a doublet or multiplet with the large molecule, additional preliminary connections could be entered into by the former, at its ends, with other amino-containing units, doublets or multiplets, and that these connections, when corresponding with those in the model, would likewise become transformed into peptide links. By an extension of this process to link after link an entire array of amino acids, in a polypeptide or protein formation mirroring that in the master molecule, would eventually become built up.

Much of the above was only implied in Delbrück's account, inasmuch as any workable hypothesis of gene copying would seem to require the idea of varied smaller units becoming somehow conjugated with like parts of the original gene and then becoming properly attached to each other so as to form an exact replica of the "parent" or "master" structure (as in the proposals made by Troland in 1917 and by the present author in 1921, *ibid.*). What commands our interest here is the chemical mechanism by which this protein is pictured as having its links forged mirrorwise. This mechanism is outlined below.

For the provision of the peptide precursor above mentioned, Delbrück starts out with Linderstrøm-Lange's as yet quite unproved proposal that the formation of peptide links proceeds indirectly, passing through several steps, beginning with amino aldehydes instead of amino acids. The aldehydes are represented as first linking spontaneously (an

advantage which amino acids do not have) to form

doublets by making a $\text{—}\overset{\text{H}}{\text{C}}=\text{N—}$ connection;

this then hydrolyzes to the form $\text{—}\overset{\text{H}}{\underset{\text{O}}{\text{C}}}\text{—}\overset{\text{H}}{\text{N—}}$,

that exists in the precursor in question.⁷ The finishing change from the latter to the real peptide linkage $\text{—}\overset{\text{H}}{\underset{\text{O}}{\text{C}}}\text{—}\text{N—}$, which is a reduction reaction

involving the loss of two H's, is a difficult step, or rather, as Delbrück puts it, series of steps, and it is for this that the aid of the preëxisting gene molecule is invoked by him.

Delbrück points out that if the precursor linkage group could get close enough (within one Å!) to the corresponding peptide linkage group in the gene—an event which he pictures as coming about in the natural course of things by thermal agitation, provided the two molecules are so constituted as to be able to fit together well—then the precursor linkage group would, on the loss of its H's (the first H loss being the only difficult one), enter into resonance with the preëxisting peptide linkage group, since they would have identical configurations with the exception of the presence of one extra electron, originally on the precursor group. Through this resonance, in which the electron tended to pass back and forth between them, a stable attachment between the two groups would be formed, which would tend to persist until, through some change in outer conditions, the two groups were released from one another, each with a mature peptide linkage in the position in question. It is apparently implied in this explanation of the loss of the H's that the very fact of the molecule without these H's, when in due juxtaposition, being able to form a stable arrangement, would be conducive to allowing that loss to be consummated. Moreover, it must be so highly conducive as to enable the loss to occur within the very limited time in which thermal agitation would leave the two groups together in just the right position.

On this hypothesis of self-duplication, as on any other, groups in the medium must be allowed to become duly attached only to like groups of the gene. Thus it is necessary to assume, in the present instance, that a given peptide-precursor and a preëxisting peptide group fit together sufficiently well when they are formed of the same amino-acids (or precursors thereof), in the same arrangement, but not when the amino-acids or their arrangement are different in the two groups. It is not evident either on ordinary chemical or on geometrical considerations why this should be the case, for no matter whether we think of the fitting as of the "front-to-front" or "front-to-back" kind, the mere fact of two complicated molecules having the same structure would not insure their also being related like lock

⁷ It is interesting to note how like a cyclol bond the connection here postulated is!

and key or mould and cast. For example, the projection of a large R group from an amino-acid should not *ipso facto* make it easier for this amino acid to fit symmetrically (no matter in which position) against another one having a similar projection. It is true that Van der Waal's forces would favor the coming together of like more than of unlike masses, but these forces, by themselves, are far from specific enough to insure the accurate like-to-like pairing here required. It might however be maintained, short of involving some unknown principle of physical science, that among the amino-acids found in the organism like and only like molecules would be able to fit one another just because only such amino acids would have been able—*ipso facto*—to engage in the processes of reproduction and growth: i.e. that there had been a sort of Democritian natural selection, leaving just such amino acids as happened to have this property. But, however this may be, Delbrück's hypothesis does not interpose any actual obstacle to the apposition of likes, and some such pairing must in any event be assumed to occur. More difficult perhaps is the question of how, on this hypothesis, the peptide-linkage groups and their corresponding precursors could get so near together as they do, by purely random movements, and even remain together long enough for the postulated change, involving the real attachment, to occur between them.

While recognizing such difficulties, it should be conceded that it is too much to require any present-day hypothesis dealing with this subject to solve at one stroke the whole complicated problem of auto-synthesis or self-copying. As Delbrück himself points out, the function of the nucleic acid, for example, remains quite unexplained on this scheme. The scheme is intended as only a partial explanation, but it has the advantage of being concrete so far as it goes, and it is very nearly the only concrete interpretation of self-duplication that has yet been offered from the side of chemistry or physics. Being so specific, it is likely that experimental means can be found of testing various matters explicitly or implicitly assumed in it: for example, the existence of oxidation-reduction reactions in the nucleus at the presumed time of gene duplication, and the existence of amino aldehydes at that time. If anything, it would seem to be too concrete, in requiring so many detailed and specific postulates simultaneously that it loses probability as a whole, but for that very reason it can the more readily be put to the test. If in this way it only serves to bring to more chemists and physicists a realization of the reality and significance of the problem of self-copying itself, and thus instigates them to do more thinking and experimentation in this direction, it will have fulfilled a highly useful purpose.

In inventing the above hypothesis, Delbrück was influenced by the consideration that no known physical forces could give the specific attraction at a distance which geneticists and cytologists have tended to infer. The hypothesis nevertheless recog-

nizes that two-by-two conjugation of likes and self-duplication are phenomena having a common basis, even though it leaves the original finding of one another by like elements to trial and error movements. I wonder, however, whether we can yet be sure of the negative proposition, that forces of specific attraction, at distances greater than the ordinary atomic ones, cannot exist between genes. Are all cases of typical synapsis simply results of a primary process of random meeting of likes, at atomic distances, at individual points in each pair of chromonemata, secondarily followed by a zipper action? If so, these points must be rather numerous, as shown by the conjugation seen in inversion loops, repeats, translocations and small chromosomes and chromosome sections in general. And are all cases of loose somatic pairing, as observed for instance in *Diptera* and in marsupials, merely "relic" states of a previous very intimate pairing of the above type, which occurred unobserved during the resting stage? Are likewise the apparent residual attractions seen where more than two threads of a kind are present—as, *par excellence*, in salivary chromosomes, even of organisms which were polyploid to start with—only relic conditions of pairings that had all been really two-by-two and at atomic distances? Are then the established cases of delayed or touch-and-go conjugation of already condensed chromosomes—like the *m* chromosomes described by Wilson, and some sex chromosomes—to be regarded as secondary phenomena, involving a non-specific long-range attraction that only superficially resembles real synapsis but has an entirely different basis? If all this may be so, nature may have played another hoax on the cytologists, and the stages in question are due for a good deal of reinvestigation, perhaps by finer means. But if specific attraction of likes at distances greater than atomic ones really occurs, then the physicists, and the biochemists as well, are due for a good shaking up. At the same time, this phenomenon, whatever its physical explanation, would go a long way in making possible self-copying by bringing the right parts together in the right positions for synthesis, as I pointed out in discussing this question in 1921 (*ibid.*) and on various occasions since that time.

A possible bearing on this question is contained in an observation which, as I mentioned in one of the Symposium discussions, was made in work on salivary chromosomes of *Drosophila* which I did in collaboration with Raffel several years ago. Here it was noticed that in a minute heterozygous inversion which we had found (that of scute-10), the homologous salivary chromosomes, although in general unable to conjugate in the inverted region (a loop being precluded by the small size of the inversion) nevertheless distinctly tended to come together at the disc nearest the midpoint of the inversion, where like loci lay opposite one another. In some cases considerable displacement or warping of parts was necessary for this. For reasons which I will not take the time to go into here, it seemed unlikely that the

conjugating elements of the middle disc were meeting with a like orientation, as might happen if they consisted of side-chains hanging onto the chromonema at only one end, or if the chromonemata were in the form of minute spirals having opposite directions in the two homologues. It was concluded instead that the like parts had conjugated even though they really lay in an inverted position with respect to one another.

From the above it was inferred, for one thing, that specific attraction of likes does occur at distances greater than atomic ones, for only the most central atom or link of all (and that in a non-matching position!) could really get into atomic range of its homologue, and it would be strange if just these had met and were holding all the rest. For another thing, since it was the dark discs, not the lighter internodes, which tended to come together, it seemed that the nucleic acid component or something associated with it played an important part in the attraction. Finally, since the spatial distribution of the constituents was opposite in the two attracting sections, it was inferred that the characteristic giving an attracting force of this kind its specificity could not be defined in terms of a spatial gradient in any plane parallel to the chromonema but must derive from the way in which the force was graduated in time, i.e. it must be an expression of some specific sort of vibration.

When I had written up the matter, in 1935, I hesitated to publish such conclusions on the basis of so isolated a case, in which there seemed so considerable a chance for special conditions to have played a role. But since that time our own observations of this case have been fully confirmed by those made by Koller working with me in Edinburgh. Moreover, figures showing the same story (though without the present deductions) have been published by Horton (1939) in her independent work on the heterozygous minute inversions found by her in the hybrid between *Drosophila simulans* and *D. melanogaster*. The fact, emerging from the latter work, that the phenomenon is so regularly to be found in such cases, disposes of the possibility (which would otherwise have been a troublesome one) that the inverted pairing might have been due merely to the presence of a reversed repeat at the middle disc, giving mirror-image figures that could conjugate with their twin parts matching.

In looking for a possible explanation of an attraction between likes based upon a like vibration of some sort, a hypothesis that had been proposed by Lamb in 1907 (J. EXP. ZOL. 5:27-33) to explain the supposed force emanating from the centrosome was recalled. Lamb has based his hypothesis on the already known fact that a body expanding and contracting periodically in a liquid attracts or repels another body with a like (but not one with an unlike) vibration, according to whether the two vibrations are in phase or not. We are not here concerned with whether Lamb was right or wrong about the centrosome, nor do we wish to main-

tain that gene attractions must have their basis in vibrations of a purely mechanical kind. But there is no doubt, as he pointed out, that like mechanical vibrations can result in specific "attraction at a distance," and it would therefore seem worth while to cast about for other possible modes of vibration or pulsation that might attain sufficient range. The possibility which naturally presented itself here was that of some kind of cyclic electronic change, or combination of changes, perhaps analogous to the oscillations occurring in crystals and metallic conductors but on a far larger scale than those hitherto encountered in the resonance of inorganic molecules or of the relatively small organic molecules ordinarily studied with reference to such questions. It seemed that the large nucleoprotein molecule might offer the opportunity for a sufficiently magnified electromagnetic or electrostatic cyclic variation. According to the very general mechanism proposed, the two particles which were attracted together would not be directly bound by a resonance in the present chemical sense—that is, by the same electrons or protons tending to alternate between them—but by the fact that the large-scale movements of electrons in one of them matched, in a complementary way, those in the other, thus giving rise, by some transfer of a part of the vibration through the intervening medium, to a pull between them. For it is evident that if there could be any kind of attractive-repulsive force at all between the particles—for instance, one of electrostatic nature conveyed through rearrangements in intervening ions or dipoles,—then if this force underwent cyclic variations in the two bodies so that they kept continually in step, thus continuing to attract one another, this attraction would be a specific one, inasmuch as two bodies with a different cycle, not being in step, would have their occasional attractions cancelled by intercalated repulsions.

Similarly, if we had a heterogeneous mixture of artificial electromagnets, floating freely about and having different frequencies of reversal of sign, those of the same frequency would be found eventually to orient towards and attract one another, specifically seeking each other out to the exclusion of the others.

After I had broached the above point of view in a tentative way to my colleagues at Edinburgh, in 1938-9, a group of papers came to hand which were so suggestively similar in their general import, and some of them backed by so much of the authority of physical science, as to give me the temerity to bring this matter up for the more public consideration of this Symposium. One of these papers was a most provocative one by Langmuir and Wrinch, published in NATURE (143:49-52) in 1939. In this the special point is elaborated upon that in a protein molecule organized into a fabric, as depicted on the cyclol theory, there would not only be opportunity for resonance between neighboring bonds—a point previously con-

tested—but the electron paths would be longer than in ordinary resonating molecules, so as to give rise to a larger scale resonance. There would of course be definite, though more or less alternative, paths for the electrons to follow, and those in neighboring tracks would to some extent influence each other. In consequence there would be certain definite frequencies set up, which in combination with each other would become synthesized into a more or less organized whole, dependent upon the entire structure of the molecule and possessed of a complicated curve. Changes in one part of a large molecule of this kind would as a result of this coördination be apt to influence very distant parts of it.

The other papers leading in the same direction began with one, somewhat earlier than the above, by Möglich and Schön (1938, *NATURWISS.* 26: 199) in which, following discussions with Timoféeff-Ressovsky and Delbrück on the large number of different atomic activations capable of producing sensibly the same mutation, the authors proposed the idea that in large protein molecules, or even groups of molecules, given electrons need not be considered as attached to individual atoms or even pairs of atoms, but might be held, as in some inorganic crystals, by an extensive "band" or network in the particle or aggregate, along with other electrons of the same energy level, all of which could be considered as interchangeable with one another. Thus the loss or gain of an electron at one point anywhere in a given band could be followed by a transference of energy occurring, in adjustment to this, at some widely distant point in the same band. The authors used this concept to explain not only the above mentioned "canalization" of the X-ray mutation reaction but also the utilization of light by chlorophyll at points distant from its absorption and other cases of distant energy transfer in organic systems. The same idea has again been expressed by Szent-Györgi in his article, "Towards a New Biochemistry," appearing in the current issue of *SCIENCE* (1941, 93: 609-611, and since then in *NATURE* 148: 157-159).

Following further discussions with Timoféeff-Ressovsky and Delbrück, and in consideration of the probable relation between gene pairing and synthesis to which the present author had repeatedly called attention (*AMER. NAT.*, 1921, *ibid.*, *SCI. MON.*, 1936, vol. 44: 210-214, and the Gene Conferences of 1936 in Copenhagen and 1938 in Spa), the physicist P. Jordan has adopted the above idea of Möglich and Schön and used it to construct a physical explanation of the supposed attraction of like parts at a distance. The core of his idea is that in the large protein molecules a kind of super-resonance would be set up which, interacting with that in an identical molecule, would result in a specific attraction between them. He has worked this out in quantum mechanical terms, and developed it in a series of articles (1938, *PHYS. Z.* 39: 711; 1939 a,

BIOL. ZBL. 59: 1-39, 1939b, *Z. f. PHYS.* 113-431; 1939c, *FUNDAM. RADIOL.* 5: 43; 1940, *Z. f. IMMUNFORSCH. U. EXP. THER.* 97: 330). It will be seen that his hypothesis, while far more definite than the intentionally general interpretation which (unknown to Jordan) had been independently postulated by me, falls within the framework provided by the latter. Pauling and Delbrück, however, have challenged the validity of Jordan's conception on grounds of the energetics of the interactions proposed by him (1940, *SCIENCE* 92: 77-79). They reason that an effective attraction could not be brought about in the given way, and calculate that unlike molecules would be affected by the postulated mechanism almost as much as like ones. They have not made it clear, however, that the same strictures would necessarily apply to any kind of cyclically varying system which the protein molecule would conceivably be capable of forming. To this extent, the lack of definiteness which is the weakness of the more general proposal which I have presented above may for a time at least serve it in lieu of strength.

For my own part, I as a mere biologist do not wish to present the above case as anything more than a tentative suggestion, for there is no use in multiplying examples of what happens to wayfarers in the regions "where angels fear to tread." But I should very much like to provoke the angels into making some more reconnaissance flights themselves over this region. The time has come when a few, at any rate, of the physicists and biochemists realize that genes and chromosomes do form valid subjects of inquiry, and that what the biologists have been saying about their mysterious properties calls for some more looking into on their part.

On their side, the investigators using purely biological methods cannot by themselves even approach a solution of the physical and chemical problems inherent in their material. But they can come across some of the problems which should be attacked by those using physical and chemical methods, and bring these problems to the attention of the latter workers; moreover, some of their results will set limits to the type of physico-chemical solution that would fit. If each of the two types of investigators would take the other more seriously than heretofore, and try to work more nearly hand in hand, the efforts of both would, in the biological field at least, be made more productive. This would seem especially true in the attack on the central problems of the gene and chromosome, as the present Symposium has demonstrated more abundantly than any previous coöperative venture in this field.

In concluding, I am expressing the intention of all the participants in offering our thanks to the organizers of this Symposium, for having for the first time focussed the efforts of so many specialists from very diverse fields upon that group of problems which is most fundamental for the understanding of the distinctive phenomena of life.

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